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Award Number: DAMD17-97-1-7152

TITLE: Immunotherapeutic Cell-Based Vaccine to Combat Metastatic
Breast Cancer

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REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20010419 052

REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Final (1 Jul 97 - 30 Jun 00)	
4. TITLE AND SUBTITLE Immunotherapeutic Cell-Based Vaccine to Combat Metastatic Breast Cancer			5. FUNDING NUMBERS DAMD17-97-1-7152	
6. AUTHOR(S) Beth Pulaski, Ph.D. S. Ostrand Rosenbert				
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Because metastases spread to many different organs and few animal models accurately reflect the clinical progression of metastatic disease, it has been difficult to develop effective therapies to specifically target these lesions. We analyzed the poorly immunogenic mouse 4T1 mammary carcinoma and demonstrated that it is an excellent model for human metastatic breast cancer. Primary 4T1 tumors displayed signs of edema, extension to the peritoneal lining, and ulcerations of the skin, which are acute diagnostic indicators of human stage IV breast cancer. More importantly, the frequency and sites of metastasis were highly comparable between 4T1 and its human counterpart. In a post-operative system, where primary 4T1 tumor is surgically removed and survival is monitored, we showed that early metastases responsible for morbidity are established by 2 weeks and that survival of these mice extends a total of 5-7 weeks. We tested novel cell-based vaccines combining MHC class II, B7.1, SEB superantigen, and IL-12 in varying protocols where wildtype spontaneous metastases were pre-established ranging from 2-4 weeks. We showed that immunotherapy initiated as late as 4 weeks effectively reduced metastatic disease. Treatment combining MHC class II ⁺ /B7.1 ⁺ /SEB ⁺ vaccines extended survival time up to 11 weeks and decreased clonogenic lung metastases by a maximum of 100-fold. Subsets of the combination were not as effective. IL-12 alone, given systemically, increased survival up to 13 weeks and reduced clonogenic lung metastases by a maximum of 1000-fold. Cell-based vaccines combined with systemic IL-12 led to 13% of the animals surviving past 27 weeks. Mechanistic studies suggest that multiple effector cells and angiostatic factors mediate the anti-tumor immune response.				
14. SUBJECT TERMS Breast Cancer, Immunotherapy, Metastases, MHC Class II, CD80 Co-stimulation, SEB superantigen, Cytokines, CD4 ⁺ and CD8 ⁺ T-lymphocytes, Angiogenesis				15. NUMBER OF PAGES 51
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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Beth L. Pulaschi 7/30/00
PI - Signature Date

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INTRODUCTION

In human breast cancer, if metastases are not present, surgical removal of the primary tumor can lead to full recovery of the patient. However, if the primary tumor has metastasized, then other therapies such as hormone (1), chemo- (2, 3), and/or radiation (4) therapy are employed to eliminate metastatic cells. In many cases these conventional treatments only lead to temporary control of the disease and provide only an average 3-year survival rate after diagnosis (5). More effective therapies are clearly necessary for treating metastatic disease. Immunologists have recently proposed and tested a variety of novel strategies for generating cell-based tumor vaccines and these approaches hold promise for additional treatment modalities. These approaches have focused on the stimulation of CD8⁺ cytotoxic T-lymphocytes (CTL), since these effector cells are capable of specifically and directly destroying malignant tumor cells. For example, various cytokine genes and/or surface molecules have been transfected into tumors and the modified tumor cells used as cell-based vaccines to enhance anti-tumor immune responses (reviewed in 6, 7). While some of these studies were designed to circumvent the need for CD4⁺ T-helper (T_h) lymphocytes by allowing the tumor cells to directly supply cytokines to CTL (6), other studies were directly aimed at increasing T_h cell generation (8, 9). Both approaches demonstrated that optimal immunity required both CD4⁺ and CD8⁺ T-cells (8-12). Most of these studies have focused on the treatment of primary tumors, and only a limited number have addressed experimental metastases (e.g. 13-16). While even fewer groups focused on established spontaneous metastatic disease, those studies used either SCID mice or anatomically incorrect tumor challenges in the footpad (17-19). Effective therapies for distant metastatic cells, therefore, have not been extensively studied and remain illusive.

T-cells recognize antigen (peptide)/major histocompatibility complexes (MHC) through their T-cell antigen receptor (TCR) (20). However, to achieve maximum activation of CD4⁺ or CD8⁺ T-cells, a second TCR-independent signal (co-stimulation) is required (21). Numerous studies have demonstrated the role of CD80 (B7.1) and CD86 (B7.2) in co-stimulation (22). Other molecules, such as ICAM-1, VCAM-1, heat stable antigen, and 4-1BB ligand (4-1BB-L) have also been shown to function in a co-stimulatory role (23-27). Although T-cell activation by superantigens (sAg) such as *Staphylococcal aureus* enterotoxin B (SEB) is not antigen specific, when complexed with MHC class II molecules on antigen presenting cells (APC), SEB is a potent polyclonal activator of CD4⁺ T-cells (28, 29). Previously, we demonstrated that the transfection of MHC class II genes into mouse sarcoma and melanoma cells enhanced primary tumor rejection

and reduced experimental (i.v.) metastases, respectively (8). Furthermore, expression of either CD80 or CD86 in addition to MHC class II increased these effects (8, 9). Not surprisingly, these responses were dependent on both CD4⁺ and CD8⁺ T-cells. Therefore, we proposed to design a mammary tumor vaccine using the tumor cells themselves to directly activate the immune system to combat spontaneous metastatic disease either before or after it becomes established. In particular, we engineered the tumor cells to express class II MHC, 4-1BB-L, and/or SEB with the expectation that these cells will activate CD4⁺ T-cells and subsequently CD8⁺ T-cells, immune effectors capable of circulating throughout the body to destroy metastatic tumor cells specifically and provide long term memory.

To test our hypotheses, we used the poorly immunogenic BALB/c mouse derived 4T1 mammary carcinoma (30-32). Our studies showed that this tumor shares many characteristics with human mammary cancers, making it an excellent animal model. Because 4T1 is resistant to the drug 6-thioguanine, micro-metastatic cells were readily detected at very early stages of growth, allowing us to more accurately quantitate the effects of our immunotherapy approach on spontaneous metastasis development. This final report summarizes the data presented in 3 published manuscripts, which have been enclosed in the Appendices (see pages 25-50), and current unpublished data. Due to the volume of the data, our published findings will be summarized briefly and referenced from the enclosed manuscripts and previously submitted Annual Reports.

BODY

Technical Objective 1: Generate mammary tumor transfectants, which can more effectively and directly present tumor antigen to CD4⁺ T-cells.

Task 1: Generating cDNA expression vectors. Genes encoding class II MHC (I-A^d, I-A^β^d), 4-1BB-L, CD80 (B7.1), and SEB were subcloned into the multiple cloning site of the pHβ-Apr-1-neo expression vector (33). A detailed description of each cDNA expression vector can be found on page 8 of 1998 Annual Summary Report (ASR).

Task 2-3: Transfection of 4T1 tumor cells. Single transfectants (4T1/A^d, 4T1/B7.1, 4T1/SEB, 4T1/4-1BB-L) and double transfectants (4T1/A^d/B7.1) were generated using the expression vectors described in task 1. Expression of MHC class I (H-2D^d), MHC class II (A^d), and B7.1 was analyzed by indirect immunofluorescent staining for the following transfectants: 4T1/A^d-1, 4T1/A^d-12, 4T1/A^d-30, 4T1/B7.1-1, 4T1/B7.1-6, 4T1/B7.1-15, 4T1/B7.1-23, 4T1/SEB-12, 4T1/SEB-14, and 4T1/A^d/B7.1-30.23 (Figure 3, Appendix page 28, and Figure 3, 1998 ASR page 10). SEB expression by 4T1/SEB-12 and 4T1/SEB-14 was determined by culturing naïve spleen cells in supernatants generated from these transfectants and measuring spleen cell proliferation (Figure 2, Appendix page 35). As previously discussed in the 1998 ASR, it was not possible to screen the 4T1/4-1BB-L transfectants due to unreliable reagents. Therefore, the 4T1/4-1BB-L cell lines were placed in cryogenic storage until such a time when they can be re-evaluated.

In summary, all necessary expression vectors were generated and transfected into 4T1 cells with the following results: 3 out of 59 clones expressed MHC class II, 6 out of 10 clones expressed B7.1 and 4 were chosen for further study based on their expression patterns, 2 of the 3 MHC class II⁺ clones (4T1/A^d-1 and 4T1/A^d-30) were double transfected with B7.1, and 2 out of 12 clones expressed SEB. While the generation of 4T1/A^d/SEB double transfectants was originally planned, it was not necessary to complete this task since mixing transfectants appeared to be effective (see tasks 11-15 below). Lastly, all of the transfectants demonstrated stable expression of the various genes and were systematically used in the following objectives.

Technical Objective 2: Characterize the immunogenicity of the mammary tumor transfectants.

Tasks 4-5: Primary tumor growth and spontaneous metastases of 4T1 parental tumor *in vivo*. Previous studies by Miller and colleagues (28-30) established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. We

confirmed these results and assessed metastatic disease in additional target organs. For a detailed description of the results see text, Figures 1-2, and Tables 1-2 on pages 26-27 in the Appendix.

Tasks 6-7: Primary tumor growth and spontaneous metastases of 4T1 tumor single transfectants *in vivo*. The tumorigenicity and spontaneous metastasis development of the 4T1/A^d and 4T1/B7.1 (Figure 4, Appendix page 29), and 4T1/SEB (Figure 8, 1998 ASR page 16) single transfectants in BALB/c mice have been described previously. With the exception of 4T1/A^d-30, all of the transfectants showed some reduction in primary tumor growth rate. A complete lack of tumorigenicity was exhibited by the 4T1/A^d-12 and 4T1/SEB-12 transfectants. In contrast, the metastatic potential of all the single transfectants was markedly reduced relative to wildtype 4T1 cells. Therefore, primary tumor growth in immunocompetent BALB/c mice is inconsistently reduced by expression of MHC class II, B7.1, or SEB genes, while metastatic disease is reproducibly decreased.

The tumorigenicity and spontaneous metastasis development of the 4T1/A^d and 4T1/B7.1 single transfectants in BALB/c *nu/nu* mice have also been described previously (Figure 5, Table 3 Appendix page 30). As shown in Figure 5, 4T1/A^d-1 and 4T1/B7.1-6 formed tumors and metastases in nude mice similar to wild type 4T1 cells. In contrast, 4T1/A^d-12 and 4T1/B7.1-23 formed primary tumor comparable to 4T1, however their metastatic potential was reduced relative to wild type 4T1 tumor cells. As summarized in Table 3, 87% of the BALB/c *nu/nu* vs. 20% of the BALB/c mice developed progressive primary tumor following s.c. challenge.

Task 8-9: Primary tumor growth and spontaneous metastases of 4T1 tumor double transfectants *in vivo*. The tumor growth and spontaneous metastases development of the 4T1/A^d/B7.1 double transfectants were not evaluated because the single transfectants exhibited a wide spectrum of *in vivo* characteristics yet uniformly were effective as cell-based vaccines (see tasks 11-15 below). Therefore, studies were immediately initiated using the double transfectants as a cell-based vaccine.

In summary, the *in vivo* analyses of the parental 4T1 tumor and the single transfectants described in technical objective 1 were completed in both BALB/c and BALB/c *nu/nu* mice. The 4T1 parental tumor appears to be an excellent model to study the effects of immunotherapy on metastatic breast cancer as our data shows that the pattern of metastatic spread is comparable to human mammary carcinoma. Assessment of the lung metastases best approximates the extent of metastatic disease in tumor-bearing mice since there is a positive correlation (correlation coefficient = 0.684) between the size of the primary tumor diameter and the number of clonogenic lung metastases (Figure 2A, Appendix page 27). All of the single transfectants exhibited a

decrease in metastatic potential and/or tumorigenicity as compared to parental 4T1. In addition, the MHC class II⁺ and B7.1⁺ transfectants display increased tumorigenicity and metastasis formation in T-cell deficient nude mice vs. immunocompetent BALB/c mice. Taken together, these results suggested that our cell-based vaccines are more immunogenic to T-cells.

Technical Objective 3: Examine the ability of the immunogenic transfectants to prevent disease and/or eliminate established spontaneous metastases.

Task 10: Radiation sensitivity of 4T1 tumor cells. Before immunizations began, the radiation sensitivity of 4T1 was determined. Cells sustained increasing dosages of radiation ranging from 0 to 50,000 rads. Proliferation was measured 48 hours after exposure to radiation by using an MTT assay and was reduced by half at doses ranging 5000 to 10,000 rads. Final analysis was performed *in vivo*, as mice were injected i.p. with 1x10⁶ parental 4T1 cells exposed to 0, 5000, 8000, or 10000 rads. Average survival time of mice injected with non-irradiated 4T1 cells was 14 days, whereas the remaining animals lived long past 45 days. In the end, a dosage of 5000 rads was chosen for immunizations described in tasks 11-15.

Tasks 11-13: Therapy Regimen One: PREVENTION- Immunization of non-tumor bearing (naive) mice prior to challenge with parental tumor. Studies using the 4T1/A^d and 4T1/B7.1 transfectants in this therapy regimen have been described previously (Figure 6, Appendix page 30). We found that immunization of naïve mice with the MHC class II⁺ and B7.1⁺ single transfectants, either alone or as a mixture, significantly protects against spontaneous metastatic disease but does not protect against primary tumor growth of the wildtype 4T1 tumor. Due to the success of the cell-based vaccines to reduce established wild type spontaneous metastatic disease (see tasks 14-15) and since our ultimate goal is to treat established spontaneous metastatic cancer, this therapy regimen was discontinued.

Tasks 14-15: Therapy Regimen Two: ESTABLISHED DISEASE- Treatment of mice bearing established 4T1 primary tumor. We have tested several combinations of our cell-based vaccines to reduce 2-3 week established spontaneous metastases in a model where the primary tumor remained in tact. We found that our 4T1/A^d and 4T1/B7.1 transfectants either alone or as a mixture were able to reduce metastatic disease that had been established for 9-14 days (Figure 7, Appendix page 31). However, the success of this treatment was limited to small tumor burdens and did not completely eliminate spontaneous metastases. We generated a more potent vaccine by adding either a cell-based vaccine encoding SEB or systemic administration of IL-12 (see Appendix pages 33-50). Although IL-12 was not originally included in the SOW, we added this

cytokine to our therapy regimen in year 2 because IL-12 and CD80 have been shown to synergize and produce optimal T-cell proliferation and IFN- γ production (34, 35) as well as stimulating primary anti-tumor responses *in vitro* (36). These results have been summarized previously (1999 ASR pages 7-8). The data is presented in Figure 4 (Appendix page 36) and Figure 3A-G (Appendix page 44), while a detailed description of the results can be found on pages 35-36 and 43 in the Appendix. To summarize briefly, therapy initiated 2-3 weeks after primary tumor inoculation reduced metastatic tumor load by a maximum of either 100-fold or 1000-fold. In addition, we found that by administering systemic IL-12 to the therapy protocol, we could reduce primary tumor growth depending on the size of the primary tumor at the time therapy began (Table 2, Appendix page 45). This affect on primary tumor growth was not observed using any of our other therapy protocols.

New Therapy Regimen: SURGERY- Post-operative 4T1 model. During the progression of this work, it became clear that the efficacy of our vaccine could not be evaluated accurately in the two therapy regimens described in the original grant proposal. The tumor vaccines have been tested for their therapeutic efficacy in a model system where the primary mammary tumor remained in tact. A potential problem with this model is that metastatic cells could continue to spread to the target organs. As a result, a third therapy regimen was developed in which the primary tumor was removed surgically, which is referred to as the post-operative model. The post-operative model has been previously described in the 1999 ASR (page 8) and a detailed description of the data can be found on page 34 and Figure 1 (page 34) in the Appendix. These results demonstrated that lethal metastasis occurs as early as 2 weeks after inoculation of primary tumor and that death following surgical removal of primary tumor resulted within 5-7 weeks (total) from outgrowth of tumor cells that metastasize early during primary tumor growth.

Treatment of mice bearing established 4T1 primary tumor in the post-operative model. We have tested several combinations of immunotherapy in the post-operative model. In the first group of surgery experiments, the tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. Therapy was initiated at week 4 (i.e. 1 week after surgery). We found that mice given injections of the cell-based vaccines (Figure 3, Appendix page 35) or systemic IL-12 (Figure 1, 1999 ASR page 9) had increased their survival rates up to 11 and 13 weeks, respectively. As discussed previously in the 1999 ASR, when animals were given a combination of IL-12 plus 4T1/A^d.B7.1+4T1/SEB as therapy, we found that the therapy itself was lethal to the animals. Therefore, we continued our experiments by testing which levels of the vaccine are safe for the animals and at the same time

remain effective against metastatic disease (Figure 1 below).

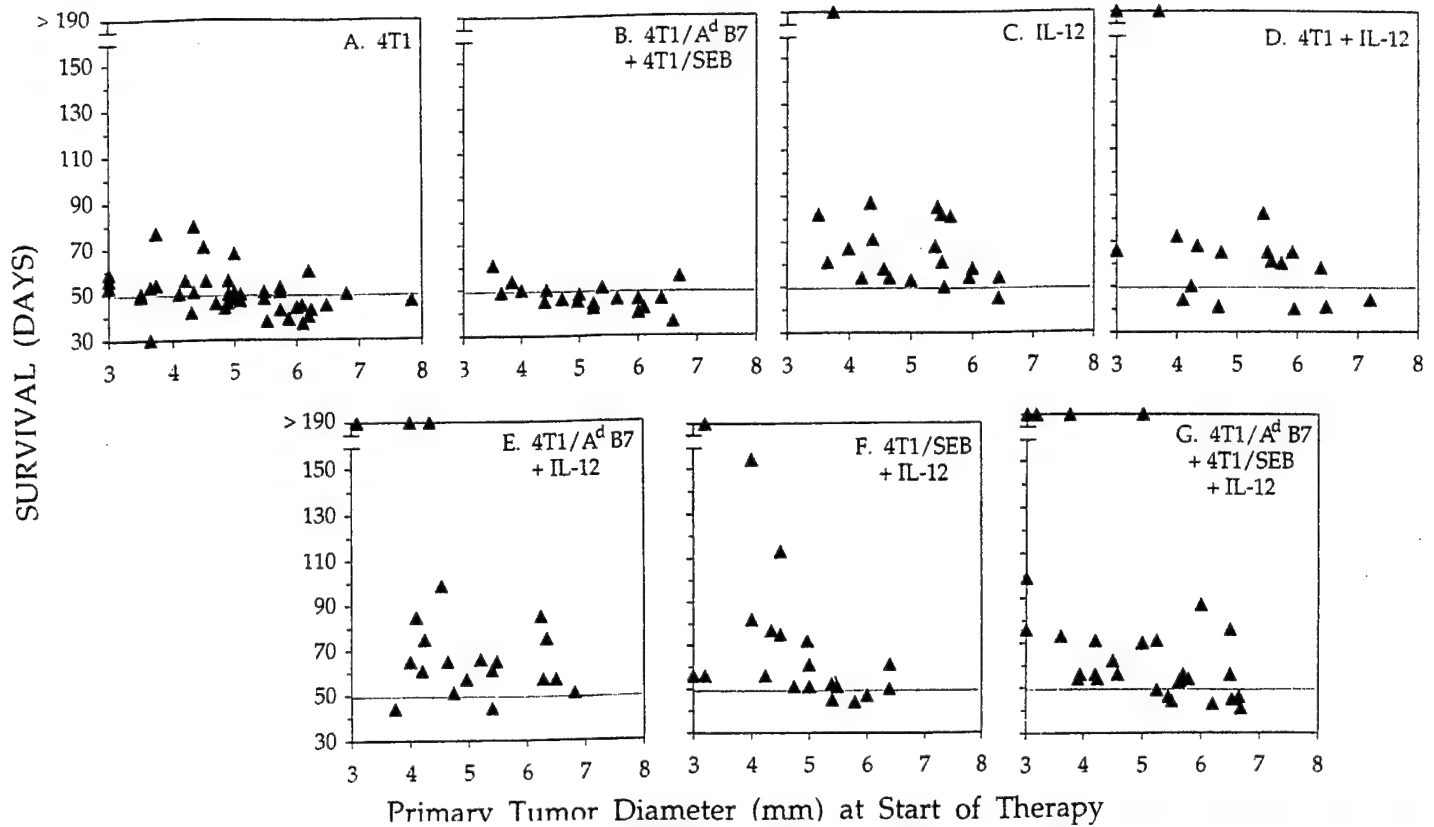


Figure 1. Cell-based vaccines plus IL-12 increases survival of mice with advanced metastatic disease. Mice were treated with therapy as described in text below with either parental 4T1 (A), 4T1/A^dB7.1 + 4T1/SEB (B), IL-12 (C), 4T1 + IL-12 (D), 4T1/A^dB7.1 + IL-12 (E), 4T1/SEB + IL-12 (F), or 4T1/A^dB7.1 + 4T1/SEB + IL-12 (G).

Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 wild type 4T1 tumor cells. On day 22, the tumor diameters were measured and animals were divided into groups based on the sizes. The average tumor diameter (mm) of primary tumors in each group ranged from $4.8 (\pm 1.0)$ to $5.2 (\pm 1.0)$. IL-12 therapy ($1 \mu\text{g}/\text{mouse}$ i.p.) was administered on days 23 and 26. Surgery was performed on day 28. Therapeutic injections of irradiated vaccine cells (1×10^6) were started on day 35 and were administered once a week for the duration of the experiment. Injections of IL-12 ($1 \mu\text{g}/\text{mouse}$ i.p.) were administered 3 times during the week following surgery and once a week thereafter. Figures 1A and 1B show that the average survival time in days for 4T1-treated and 4T1/SEB + 4T1/A^dB7.1-treated animals was $49.8 (\pm 8.3)$ and $47.2 (\pm 5.6)$, respectively. Treatment with IL-12 alone (Figure 1C), 4T1 + IL-12 (Figure 1D), or 4T1/SEB + IL-12 (Figure 1F) resulted in 1/20 (5%), 2/18 (11%), or 1/20 (5%) animals, respectively, surviving past 190 days (27 weeks). The greatest increase in survival was seen in the mice treated with

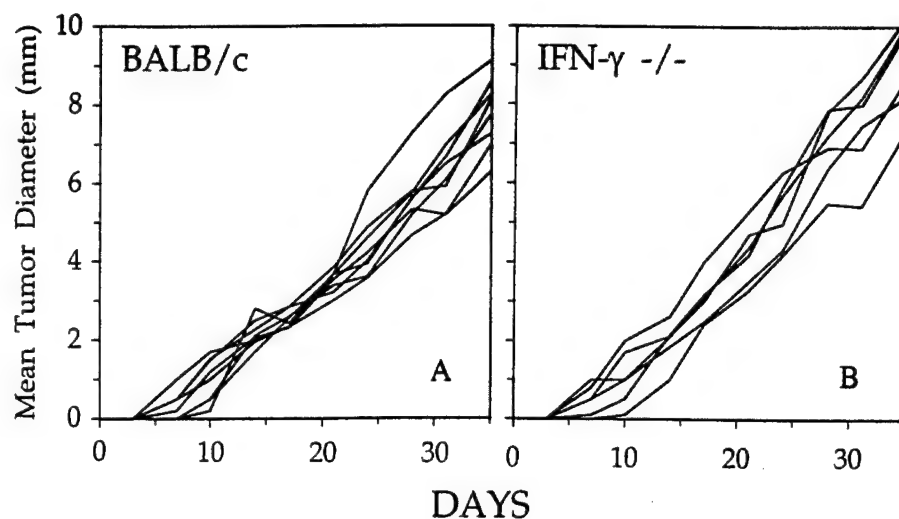
either 4T1/A^d/B7.1 + IL-12 (Figure 1E) or 4T1/A^d/B7.1 + 4T1/SEB + IL-12 (Figure 1G) which resulted in 3/21 (14%) and 4/32 (13%) animals surviving past 190 days (27 weeks).

Task 16: Analysis of immune response mechanism. The concept of combining SEB and IL-12 with MHC class II and CD80 was based on the hypothesis that SEB and IL-12 are potent activators of CD4⁺ T lymphocytes (28, 29, 37) and would provide additional activation signals. Therefore, to understand the mechanism of vaccine efficacy and demonstrate that T-cells are involved in the immune response against metastatic cancer, we tested the immunotherapy protocols in GK1.5 (CD4), 2.43 (CD8), or anti-asialo-GM1 (NK) antibody-depleted animals as well as BALB/c *nu/nu* and *beige/nude/XID* mice. Depletion of CD4⁺ or CD8⁺ T cells eliminates the therapeutic effect of the MHC class II⁺, B7.1⁺, SEB⁺ vaccine against spontaneous metastases, while depletion with control ascites does not (Figure 5A-D, Appendix page 37). In addition, the combination vaccine does not reduce metastatic disease in BALB/c *nu/nu* mice, which are deficient for T cells (Figure 5E-F, Appendix page 37). To our surprise, depletion of CD4⁺, CD8⁺ T cells, and/or NK cells did not diminish the therapeutic effect the IL-12 based treatments (see Figures 3H-K and 4, Appendix pages 44-46 for a detailed description of the results). Therefore, other effector mechanisms are most likely involved.

Additional Mechanistic Studies. Recent studies indicate that IL-12 and its downstream mediator IFN- γ may regulate tumor growth by stimulating anti-angiogenic chemokines including Monokine Induced by IFN- γ (Mig) and IFN- γ Inducible Protein 10 (IP-10) (38-40). To determine if our IL-12 therapy involves Mig and/or IP-10, RNA was prepared from the lungs of tumor-bearing therapy mice, reverse-transcribed, and PCR amplified using Mig-specific and IP-10-specific PCR primers. Although IP-10 mRNA was not expressed in the lungs, Mig mRNA was present in the lungs of 4T1 tumor-bearing mice receiving immunotherapy. Mig mRNA was detected as early as 4 hours after initiation of therapy and throughout the 21-day duration of therapy (Figure 5C, Appendix page 47). Control untreated, tumor-free mice did not express Mig in their lungs (Figure 5A, Appendix page 47). We further demonstrated that 4T1 synthesizes Mig when co-cultured with IFN- γ for 2 hours *in vitro* (Figure 6, Appendix page 48) which suggested that 4T1 metastatic tumor cells were responsible for the production of Mig *in vivo*.

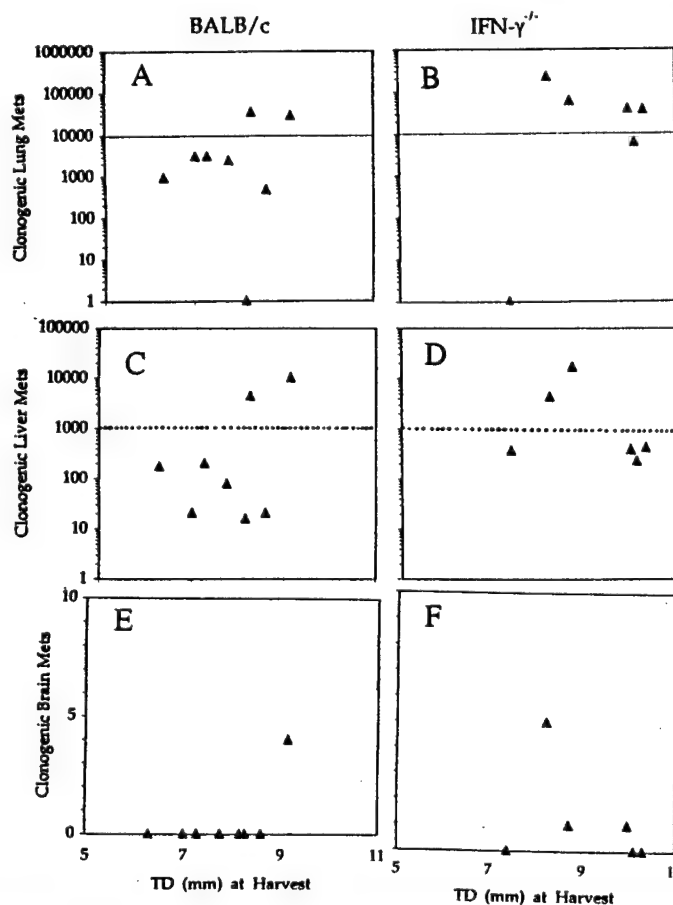
Since the publication of these results, we have performed several experiments to examine the role of IFN- γ in our system. The tumorigenicity and metastatic potential of 4T1 has been measured in IFN- γ knockout mice (IFN- γ ^{-/-}) and compared to the kinetics found in BALB/c mice. Primary tumors form in 100% of IFN- γ ^{-/-} and BALB/c mice when challenged s.c. in the abdominal mammary gland with as few as 7x10³ 4T1 cells (Figure 2). These tumors are palpable

Figure 2: 4T1 cells are highly tumorigenic in IFN- γ ^{-/-} and BALB/c mice. Female BALB/c (A) (n=8) and IFN- γ ^{-/-} (B) (n=6) mice were challenged s.c. in the abdominal mammary gland with 7×10^3 parental 4T1 cells. Primary tumors were measured every 3-4 days and the mean tumor diameter (TD) was calculated as the square root of the product of 2 perpendicular diameters. Each line represents individual mouse.



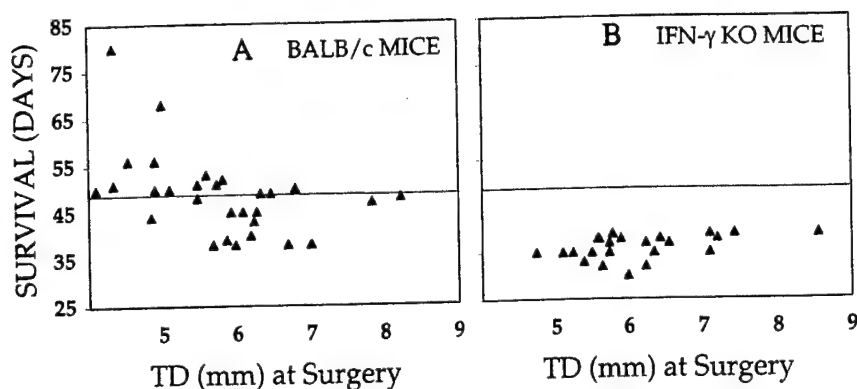
in both groups of mice within 10 days, however, tumor growth is slightly increased in IFN- γ ^{-/-} mice. At day 35, the average tumor diameter is $9.1 (\pm 0.9)$ and $7.8 (\pm 1.4)$ in IFN- γ ^{-/-} versus BALB/c mice ($p < 0.05$, student's two-tailed t-test). In addition, metastatic disease is dramatically increased in IFN- γ ^{-/-} mice (Figure 3) and, subsequently, coincides with decreased survival of these mice (Figure 4) as compared to BALB/c mice. By day 35, 67% (4/6) IFN- γ ^{-/-} (Figure 3B) and 25% (2/8) BALB/c (Figure 3A) mice have $>10^4$ clonogenic lung metastases. Clonogenic liver (Figures 3C versus 3D) and brain (Figures 3E versus 3F) metastases were similarly increased in IFN- γ ^{-/-} mice.

Figure 3: Metastatic disease is significantly increased in IFN- γ ^{-/-} mice. Female BALB/c (A, C, E) (n=8) and IFN- γ ^{-/-} (B, D, F) (n=6) mice were challenged in the abdominal mammary gland s.c. with 7×10^3 parental 4T1 cells. On day 35, the lungs (A, B) liver (C, D) and brain (E, F) were harvested and analyzed for the number of clonogenic metastatic cells present. Each point represents an individual mouse.



We observed the most dramatic difference between the IFN- γ ^{-/-} and BALB/c mice when measuring survival (Figure 4). The average survival time of IFN- γ ^{-/-} and BALB/c mice challenged with 4T1 tumor was 36.0 (\pm 6.2) and 49.7 days, respectively ($p < 0.01$, student's two-tailed t-test). Therefore, IFN- γ appears to be an important regulator of tumor growth *in vivo*.

Figure 4: Survival of IFN- γ ^{-/-} mice bearing 4T1 tumor is significantly decreased. Female BALB/c (A) (n=34) and IFN- γ ^{-/-} (B) (n=23) mice were challenged s.c. in the abdominal mammary gland with 7×10^3 parental 4T1 cells. Primary tumors were measured and surgically removed on day 24. Each point represents the survival time in days after the primary tumor challenge for individual mouse.



Future Directions. More experiments need to be done to examine the role of IFN- γ , in particular, as well as the effector mechanisms responsible for the enhanced immune response against metastatic disease in our system. In addition to T-cells, NK and macrophages may also be playing a role in the therapeutic efficacy of our vaccines. Each of these populations need to be more closely studied for their role in innate immune responses to the tumor itself (tasks 4-5) as well as their role in the immunizations (tasks 11-15). The primary focus of future studies should identify the effector mechanisms responsible for the reduction in metastatic tumor because only then will we be able to appropriately modify the vaccine and increase survival further. Because there is a significant difference in the efficacy of our vaccines to eliminate primary tumor versus metastatic disease, a new direction of experiments that compare and contrast the immune responses at these sites should be pursued and could lead to a better understanding of how to treat metastatic breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

1. 4T1 is an excellent animal model for human breast cancer.
 - Metastasis sites are common between human breast cancer and 4T1: spreading first to the lungs and liver in 24-77% and 22-62% of women, respectively, versus >95% and >75% of BALB/c mice, respectively. Metastasis to the central nervous system is characteristically less frequent for both humans and the 4T1 tumor (30% and 40%, respectively) and statistically occurs later in the disease process.
 - The 4T1 tumor can be used as a post-operative model, where primary tumor is surgically removed and survival is monitored.
 - Early metastases established by 2-weeks after tumor inoculation with 7×10^3 wildtype 4T1 cells are responsible for animal morbidity and survival of these mice extends a total of 5-7 weeks.
 - Primary 4T1 tumors show signs of edema, extension to the peritoneal lining, and ulcerations of the skin at the time of surgery, which are acute diagnostic factors of human stage IV breast cancer.
2. Treatment of 2-week established tumor-bearing mice with transfectants expressing MHC Class II, B7.1, and SEB reduces established wild type metastatic cancer by a maximum of 100-fold.
3. Mice carrying 3-week established 4T1 mammary carcinoma metastases have reductions in metastatic disease up to a maximum of 1000-fold following treatment with IL-12 plus 4T1 transfectants (4T1/A^d and 4T1/B7.1) or wild type tumor.
4. Combinations of IL-12 and cellular therapy reduce growth of small primary solid tumors.
5. Treatment of 4-week established wild type metastases in post-operative mice with transfectants expressing MHC Class II, B7.1, and SEB increases survival up to 11 weeks.
6. Treatment of 4-week established wild type metastases in post-operative mice with IL-12 increases survival up to 13 weeks.
7. Combining MHC class II⁺, B7.1⁺, and SEB⁺ vaccines with systemic IL-12 in the post-operative model leads to 13% (4/32) animals surviving past 190 days (27 weeks).
8. Reduction of established wild type metastases with MHC class II, B7.1, and SEB immunotherapy requires both CD4⁺ and CD8⁺ T cells.
9. IL-12 immunotherapeutic effect is not exclusively dependent on CD4⁺, CD8⁺, or NK cells.
10. IFN- γ and Mig may play an important role in controlling primary and metastatic disease.

REPORTABLE OUTCOMES

Manuscripts

1. Pulaski, B.A., & Ostrand-Rosenberg, S. (1998) Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Res.*, 58: 1486-1493.
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7. Pulaski, B.A., Clements, V.K., Pipeling, M.R., & Ostrand-Rosenberg, S. (2000) Immunotherapy combining MHC class II/CD80-cell based vaccines with IL-12 reduces established metastatic disease and stimulated immune effectors and monokine-induced by interferon- γ . *Cancer Immunol. Immunother.*, 49: 34-45.
8. Pulaski, B.A., Terman, D.S., Khan, S., Muller, E., & Ostrand-Rosenberg, S. (2000) Cooperativity of SEB superantigen, MHC class II, and CD80 for immunotherapy of advanced metastases in a clinically relevant post-operative mouse breast cancer model. *Cancer Res.*, 60: 2710-2715.
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Abstracts

1. Ostrand-Rosenberg, S., & Pulaski, B.A. Control of metastatic mammary cancer following immunotherapy with gene modified tumor cells. An Era of Hope, Dept. of Defense Breast Cancer Program Meeting, Nov., 1997.
2. Pulaski, B.A., & Ostrand-Rosenberg, S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with MHC class II and B7.1 cell-based tumor vaccines. A Look Ahead: Breakthrough Innovations in the Search for Healthier Lives, Nov., 1997.
3. Pulaski, B.A., & Ostrand-Rosenberg, S. Immunotherapy with MHC class II and B7.1 cell-based tumor vaccines reduce established spontaneous mammary carcinoma metastases. Society for Leukocyte Biology, 32nd National Meeting, Dec., 1997.
4. Pulaski, B.A., Terman, D., Khan, S., Muller, E., & Ostrand-Rosenberg, S. Synergy of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer. 90th Annual Meeting of the AACR, April, 1999.
5. Pulaski, B.A., Terman, D., Khan, S., Muller, E., & Ostrand-Rosenberg, S. Synergy of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer. Experimental Biology '99, April, 1999.
6. Pulaski, B., Terman, D., Khan, S., Muller, E., & Ostrand-Rosenberg, S. Synergy of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer. Cancer Immunossurveillance 1999, Oct., 1999.
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8. Pulaski, B.A., & Ostrand-Rosenberg, S. MHC class II, B7.1, SEB, and IL-12 immunotherapy eliminates spontaneous mammary metastases in a clinically relevant mouse model. Cellular Immunity and Immunotherapy of Cancer, Jan., 2000.
9. Pulaski, B.A., & Ostrand-Rosenberg, S. MHC class II, B7.1, SEB, and IL-12 immunotherapy eliminates spontaneous mammary metastases in clinically relevant mouse model. Era of New Hope Hope, Dept. of Defense Breast Cancer Program Meeting, June, 2000.

Oral Presentations

1. Pulaski, B.A. Immunotherapy with MHC class II and B7.1 cell-based tumor vaccines reduce established spontaneous mammary carcinoma metastases. Society for Leukocyte Biology, 32nd National Meeting, Late-Breaking Abstracts, Dec. 6, 1997.
2. Pulaski, B.A., Terman, D., Khan, S., Muller, E., & Ostrand-Rosenberg, S. Synergy of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced spontaneous metastatic breast cancer. Experimental Biology '99, Block Symposium: Tumor Vaccines, April 18, 1999.
3. Pulaski, B.A. IL-12 synergizes with MHC class II/B7.1-cell based vaccines to stimulate immune effectors and anti-angiostatic mechanisms that reduce established metastatic disease. 14th Annual Scientific Meeting of the Society for Biological Therapy, Presidential Award Session, Oct. 29, 1999.
4. Pulaski, B.A. MHC class II, B7.1, SEB, and IL-12 immunotherapy eliminates spontaneous mammary metastases in clinically relevant mouse model. Era of New Hope, Dept. of Defense Breast Cancer Program Meeting, General Session: Invasion and Metastasis, June 11, 2000.

Cell Lines

1. The 4T1 tumor cell line is currently being deposited to American Type Cell Collection (Rockville, MD).

Funding applied for based on this work

1. Title: Immunotherapy and immunosuppression of metastatic mammary cancer
Supporting Agency: DOD, US AMRMC Breast Cancer Program (CDA Award)
Duration/Level of Funding: 03/01/01-02/28/05; \$333,110
2. Title: Immunotherapy and immunosuppression of metastatic mammary cancer
Supporting Agency: DOD, US AMRMC Breast Cancer Program (IDEA Award)
Duration/Level of Funding: 03/01/01-02/28/04; \$434,021
3. Title: Immunotherapy and immunosuppression of metastatic mammary cancer
Supporting Agency: Komen Foundation for Breast Cancer Research
Duration/Level of Funding: 09-01-01 to 08-30-03; \$250,000

4. Title: Immunotherapy and immunosuppression of metastatic mammary cancer

Supporting Agency: ACS beginning investigators award

Duration/Level of Funding: Startup money, \$25,000

5. Title: Role of T cells and chemokines in metastatic cancer

Supporting Agency: NIH (K01) The Howard Temin Award

Duration/Level of Funding: 07-01-01 to 08-30-05; \$480,000

Employment Opportunities and Experiences applied for and/or received

1997-2000 Associate member of American Association for Cancer Research

March 2000 Assistant Research Scientist, Dept. of Biological Sciences, University of Maryland
Baltimore County

June 2000 Appointment to the Program in Oncology, University of Maryland Greenebaum
Cancer Center

CONCLUSIONS

A detailed discussion of the results can be found on pages 29-31, 36-38, and 47-49 in the Appendix.

We can conclude from these results that tumor-cell based immunotherapy is a viable alternative option for the treatment of metastatic breast cancer. Figure 5 summarizes our "working" hypothesis. Although our goal was to generate a vaccine that enhanced T-cell activation, we believe that multiple effector mechanisms are being upregulated by the immunotherapy. It appears that MHC class II, CD80, and SEB are stimulating a classical T-cell response. However, the addition of IL-12 to the immunotherapy appears to up-regulate non-T-cell pathways. For example, we think that IL-12 enhances NK cell activation and anti-angiogenic cytokine(chemokine) production *in vivo*. We now believe that the stimulation of all three pathways is important for the elimination of metastatic disease because survival is maximally increased when all of the factors (MHC class II, CD80, SEB, and IL-12) are combined. We believe that vaccines designed to activate multiple pathways will lead to the best immunotherapy treatments. As a result of our IFN- γ experiments (Figures 2-4), we now see that innate immune responses may also play an important role in controlling tumor growth and future work should include studies to understand these mechanisms.

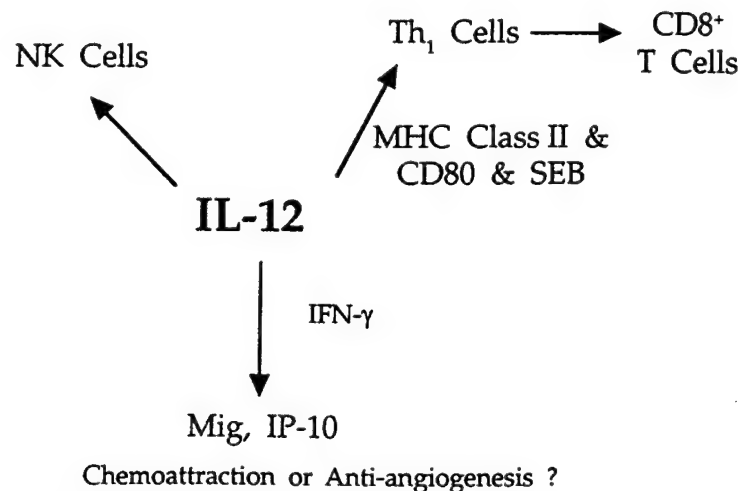


Figure 5: Proposed mechanisms of action by the MHC class II, CD80, SEB, and IL-12 immunotherapy. MHC class II, CD80, and SEB work co-operatively to activate classical T-cells, while IL-12 stimulates independent mechanisms that complement the T-cell activation pathway.

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Reduction of Established Spontaneous Mammary Carcinoma Metastases following Immunotherapy with Major Histocompatibility Complex Class II and B7.1 Cell-based Tumor Vaccines¹

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ABSTRACT

For many cancer patients, removal of primary tumor is curative; however, if metastatic lesions exist and are not responsive to treatment, survival is limited. Although immunotherapy is actively being tested in animal models against primary tumors and experimental metastases (i.v. induced), very few studies have examined immunotherapy of spontaneous, established metastatic disease. The shortage of such studies can be attributed to the paucity of adequate animal models and to the concern that multiple metastatic lesions may be more resistant to immunotherapy than a localized primary tumor. Here, we use the BALB/c-derived mouse mammary carcinoma, 4T1, and show that this tumor very closely models human breast cancer in its immunogenicity, metastatic properties, and growth characteristics. Therapy studies demonstrate that treatment of mice with established primary and metastatic disease with MHC class II and B7.1-transfected tumor cells reduces or eliminates established spontaneous metastases but has no impact on primary tumor growth. These studies indicate that cell-based vaccines targeting the activation of CD4⁺ and CD8⁺ T cells may be effective agents for the treatment of malignancies, such as breast cancer, where the primary tumor is curable by conventional methods, but metastatic lesions remain refractile to current treatment modalities.

INTRODUCTION

In human breast cancer, if metastases are not present, surgical removal of the primary tumor can lead to full recovery of the patient. However, if the primary tumor has metastasized, then other therapies such as hormone therapy (1), chemotherapy (2, 3), and/or radiation therapy (4) are used to eliminate metastatic cells. In many cases, these conventional treatments only lead to temporary control of the disease and provide only an average 3-year survival rate postdiagnosis (5). More effective therapies are clearly necessary for treating metastatic disease. Immunologists have recently proposed and tested a variety of novel strategies for generating cell-based tumor vaccines, and these approaches hold promise for additional treatment modalities. These approaches have focused on the stimulation of CD8⁺ CTLs because these effector cells are capable of specifically and directly destroying malignant tumor cells. For example, various cytokine genes and/or surface molecules have been transfected into tumors, and the modified tumor cells have been used as cell-based vaccines to enhance antitumor immune responses (reviewed in Refs. 6 and 7). Although some of these studies were designed to circumvent the need for CD4⁺ T_H³ lymphocytes by allowing the tumor cells to directly supply cytokines

to CTLs (6), other studies were directly aimed at increasing T_H cell generation (8, 9). Both approaches demonstrated that optimal immunity required both CD4⁺ and CD8⁺ T cells (8-12). Most of these studies have focused on the treatment of primary tumors, and only a limited number have addressed experimental metastases (e.g., Refs. 13-16). Although even fewer groups focused on established spontaneous metastatic disease, those studies used either severe combined immunodeficient mice or anatomically incorrect tumor challenges in the footpad (17-19). Effective therapies for distant metastatic cells, therefore, have not been extensively studied and remain elusive.

T cells recognize antigen (peptide)/MHCs through their T-cell antigen receptor (20). However, to achieve maximum activation of CD4⁺ or CD8⁺ T-cells, a second T-cell antigen receptor-independent signal (costimulation) is required (21). Numerous studies have demonstrated the role of B7.1 and B7.2 in costimulation (22). Other molecules, such as intercellular adhesion molecule-1, VCAM-1, heat-stable antigen, and 4-1BB ligand have also been shown to function in a costimulatory role (23-27). Previously, we demonstrated that the transfection of MHC class II genes into mouse sarcoma and melanoma cells enhanced primary tumor rejection and reduced experimental (i.v.) metastases, respectively (8). Furthermore, expression of either B7.1 or B7.2 in addition to MHC class II increased these effects (8, 9). Not surprisingly, these responses were dependent on both CD4⁺ and CD8⁺ T cells. We now propose that by designing tumor cells as vaccination vehicles for stimulating both CD4⁺ and CD8⁺ T-cells, it should be possible to induce tumor-specific immunity to treat spontaneous metastatic disease.

To test this hypothesis, we have used the poorly immunogenic BALB/c mouse-derived 4T1 mammary carcinoma (28-30). This tumor shares many characteristics with human mammary cancers, making it an excellent animal model, and it expresses adequate levels of MHC class I molecules, making it a suitable target for CD8⁺ T cells. Because 4T1 is 6-thioguanine resistant, micrometastatic cells can readily be detected at very early stages of growth, allowing us to quantitatively monitor the effects of the immunotherapy approach on spontaneous metastasis development.

MATERIALS AND METHODS

Animals and Reagents. Female BALB/c and BALB/c *nu/nu* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and were used at 8 weeks of age. Reagents were purchased as indicated: Lipofectin and G-418 sulfate (Geneticin; Life Technologies, Inc., Gaithersburg, MD); collagenase types 1 and 4 (Worthington Biochemical Corp., Freehold, NJ); elastase (ICN, Costa Mesa, CA); hyaluronidase, BSA, 6-thioguanine (2-amino-6-mercaptopurine), and methylene blue, Sigma Chemical Co. (St. Louis, MO).

cDNA Expression Vectors. The expression vector pHβ-Ap1-neo has been described previously (31). Using PCR, cDNAs encoding the A_α^d and A_β^d class II MHC genes were amplified from RNA isolated from A20 B-lymphoma cell line. Primers for the A_α^d chain (sense, 5'-CTCCGCGAGTCGACGAT-GCCGTGCAGCAGA-3'; and antisense, 5'-ACAGCGGATCCTCATAAAG-GCCCTG-3') and A_β^d chain (sense, 5'-CCTGTGCAGTCGACATGGCTCT-GCAGAT-3'; and antisense, 5'-GACACGGATCCTCACTGCAG GAGCC-3') incorporated a *Sall* site at their 5' end and a *Bam*HI site at their 3' end for

Received 11/3/97; accepted 1/29/98.

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¹ This work was supported by United States Army Research and Development Command Grant DAMD17-94-J-4323, NIH Grant RO1 CA52527, and United States Army Research and Development Command Postdoctoral Fellowship DAMD17-97-1-7152 (to B. A. P.).

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³ The abbreviations used are: T_H, T-helper; TD, mean tumor diameter; LN, lymph node; CC, correlation coefficient; NK, natural killer; APC, antigen-presenting cell; mAb, monoclonal antibody.

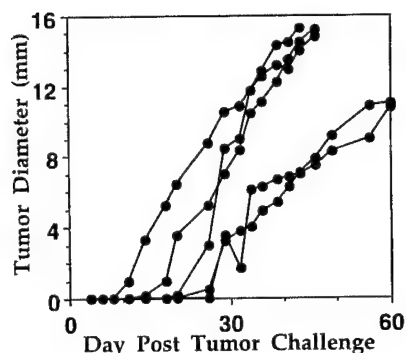


Fig. 1. 4T1 cells are highly tumorigenic. Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Primary tumors were measured every 3–4 days, and the mean TD was calculated as described in "Materials and Methods." Lines, individual mice.

subcloning into the parental vector. The expression vector containing the B7.1 cDNA was also generated using PCR and was described previously (32). The final constructs contained only the sequence within the coding region for each cDNA and conferred resistance to G-418.

Cell Lines and Transfectants. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma, was kindly supplied by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI; Ref. 30). Unmodified tumor cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine product (Hyclone, Logan, UT) and $1 \times$ antibiotic-antimycotic (Life Technologies, Inc.). Transfectants were made to express either MHC class II or B7.1 by using Lipofectin according to the manufacturer's instructions. Cells were selected with $400 \mu\text{g}/\text{ml}$ G-418, cloned by limiting dilution 48 h after transfection, stained for surface antigen expression, and analyzed by flow cytometry as described previously (8, 9). The following antibodies were used: 34-5-8, mouse anti-H-2D^d (33); 16.3.1, mouse anti-H-2K^k (34); MKD6, mouse anti-I-A^d (35); 3JP, mouse anti-I-A^{b,k} (36); and IG10, rat anti-B7.1 (37).

In Vivo Tumor Growth. Mice were challenged s.c. in the abdominal mammary gland with either parental or transfectant 4T1 tumor cells. Primary tumors were measured every 3 or 4 days following tumor challenge using vernier calipers. Mean TD was calculated as the square root of the product of two perpendicular diameters. Animals were sacrificed when the TD reached 14–16 mm or when the mice became moribund, according to University of Maryland Baltimore County Institutional Animal Care and Use Committee guidelines.

Spontaneous Metastases Assay. Spontaneous metastases were measured by adapting methods described previously by Aslakson and Miller (30). Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental or transfectant 4T1 tumor cells and sacrificed at the times indicated. Several organs were removed from each mouse, uniquely identified, and further prepared as follows: Blood and draining LNs were prepared as described previously (30). Liver samples were finely minced and digested in 5 ml of enzyme cocktail containing $1 \times$ PBS, 0.01% BSA, 1 mg/ml hyaluronidase, and 1 mg/ml collagenase type 1 for 20 min at 37°C on a platform rocker. Lung samples were finely minced and digested in 5 ml of enzyme cocktail containing $1 \times$ PBS, 1 mg/ml collagenase type 4 and 6 units/ml elastase for 1 h at 4°C on a rotating wheel. Brain samples were finely minced and digested for 2 h at 37°C on a platform rocker with 5 ml of the same enzyme cocktail used for lung samples. After incubation, all samples were filtered through $70\text{-}\mu\text{m}$ nylon cell strainers and washed two to three times with $1 \times$ HBSS. Resulting cells were resuspended and plated neat or serially diluted in 10-cm tissue culture dishes in medium containing $60 \mu\text{M}$ thioguanine for clonogenic growth. 6-Thioguanine-resistant tumor cells formed foci within 10–14 days, at which time they were fixed with methanol and stained with 0.03% methylene blue for counting. Clonogenic metastases were calculated on a per-organ basis.

Statistical Analyses. A Student's *t* test for unequal variances was performed using Microsoft Excel Version 5.0 to determine the statistical significance of indicated data.

RESULTS

Inoculation of Small Quantities of 4T1 Mammary Carcinoma Induces Primary Tumor Formation and Spontaneous Metastatic Disease in Syngeneic BALB/c Mice. Previous studies by Miller and colleagues (29, 30) and others (28) established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. Because we are developing immunotherapy strategies for the treatment of metastatic malignancies, we have confirmed these results and assessed metastatic disease in additional target organs as a prelude to our therapeutic studies. As shown in Fig. 1 and Table 1, primary tumors form in 100% of BALB/c mice when as few as 5×10^3 cells are injected s.c. in the abdominal mammary gland. These tumors are palpable within 11–26 days after injection and reach 14–16 mm in TD within 40–69 days. At higher doses ($>10^4$), primary tumors develop more rapidly, as reflected in a shortened tumor onset and decreased survival time. Although inoculation of lower doses of 4T1 (10^3) also induces primary tumor formation, the tumor incidence decreases to 60% of inoculated mice. The 4T1 tumor, therefore, is highly tumorigenic, even at relatively low doses of inoculating cells.

To confirm the metastatic potential of the 4T1 mammary carcinoma, female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 4T1 cells, and metastasis formation was assessed. Mice were sacrificed at varying times after inoculation and the kinetics of spontaneous metastasis formation were assessed in the draining LN, lung, liver, blood, and brain by plating out dissociated organs in medium supplemented with 6-thioguanine. Because 4T1 cells are 6-thioguanine resistant, individual tumor cells form foci in culture, each focus representing an individual clonogenic tumor cell. The number of foci, therefore, is a direct measure of the number of metastatic tumor cells per organ, and the *in vitro* amplification allows for the quantitation of micrometastatic tumor cells, which would otherwise not be detectable.

Table 2 shows the distribution and subsequent spread of metastatic tumor cells in the various organs at progressive times after inoculation. For example, at day 14 or 18 after primary s.c. inoculation, distant spontaneous metastases were measurable in the LN of 11 of 12 mice and the lungs of 13 of 13 mice. By day 22, the livers of three of five mice had clonogenic metastases, whereas the blood of only one of eight mice contained tumor cells. Because only a portion of the blood was recovered, this value may be an underestimate. By week 4, the blood, liver, and lungs of 75–100% of mice contained tumor cells. Some of the organs with clonogenic tumor cells showed visible metastatic lesions; however, many of the organs appeared phenotypically normal and showed no visible signs of tumor. Also by week 4, the draining LN of five of eight mice had been engulfed by the primary tumor and, thus, could not be tested. Metastatic cells in the brain were first detected at week 5 (27% of mice) and the frequency of mice with metastatic cells in the brain increased (67%) as time progressed. Metastases in the blood, LN, liver, and/or brain of indi-

Table 1 Tumor growth analysis of 4T1 mammary carcinoma in syngeneic BALB/c mice

BALB/c mice (five mice/group) were challenged s.c. in the abdominal mammary gland with the indicated number of parental 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Challenge dose	Tumor incidence	Tumor onset (days)	Time to sacrifice (days)
1×10^3	3/5	15–20	45–61
5×10^3	5/5	11–26	40–69
1×10^4	5/5	8–10	35–46
1×10^5	5/5	6–8	35
1×10^6	5/5	4–7	30

Table 2 4T1 mammary carcinoma cells spontaneously metastasize in BALB/c mice

BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 tumor cells. Mice were sacrificed at various times after tumor challenge, and the draining lymph node, lung, liver, blood, and brain tissues were removed. Each organ was individually prepared as described in "Materials and Methods" and plated for metastatic cell outgrowth. Data indicate the number of animals positive for spontaneous metastases of the total number tested for each organ. The numbers in parentheses show the range of clonogenic metastases found in the positive organs.

Harvest day	Spontaneous metastases				
	LN	Lung	Liver	Blood	Brain
14-18	11/12 (2-57)	13/13 (1-43)	0/11	0/13	ND ^a
22	7/9 (5-35)	6/11 (32-338)	3/5 (1)	1/8 (1)	ND
30-32	2/3 (15-83)	10/10 (6-116,500)	7/8 (7-3,700)	3/4 (6-82)	ND
34-37	ND	10/12 (315-267,000)	11/14 (32-7,800)	5/11 (1-24)	3/11 (1-116)
>42	ND	14/14 (1,109-200,000)	6/8 (1,100-12,200)	6/8 (25-490)	4/6 (5-613)

^a ND, not done.

vidual mice were only present when the individual contained lung metastases and not *vice versa*. The pathway of metastasis for the 4T1 tumor, therefore, appears to be from the primary tumor to the lungs and the draining LN and, subsequently, to the liver, blood, and brain.

There is frequently a correlation in human disease between the size of primary tumor and extent of metastatic disease. To determine whether this observation is modeled by the 4T1 tumor, the number of clonogenic tumor cells in the lung, liver, blood, LN, and brain has been plotted as a function of the TD at the time of harvest. As shown in Fig. 2A, there is a positive correlation ($CC = 0.684$) between size of primary tumor at time of sacrifice and the number of clonogenic lung metastases. Similar correlations between TD at the time of harvest and clonogenic metastases were also seen for liver (Fig. 2B, $CC = 0.520$), blood (Fig. 2C, $CC = 0.396$), and brain (Fig. 2D, $CC = 0.426$). No correlation was seen between the number of clonogenic metastases in LN and the size of primary tumor (Fig. 2E, $CC = 0.134$) because the number of samples were limiting. The 4T1 tumor, therefore, shows a pattern of metastatic spread comparable to

human mammary carcinoma, and assessment of lung metastases best approximates the extent of metastatic disease in tumor-bearing mice.

Expression of MHC Class II or B7.1 by 4T1 Transfectants Reduces Tumorigenicity and Metastatic Potential. In previous studies, we demonstrated that sarcoma cells transfected with syngeneic MHC class II plus B7.1 genes are an effective cell-based vaccine for the treatment of established, primary, solid tumors (9). That strategy was based on the hypothesis that such vaccines could activate both CD4⁺ and CD8⁺ tumor-specific T-cells and that optimal activation of CD8⁺ T-cells requires "help" from CD4⁺ T-cells. Because such vaccines might be very desirable agents for the treatment of disseminated metastatic disease, we have now extended our studies to the spontaneously metastatic 4T1 breast carcinoma.

4T1 tumor cells were transfected with plasmids containing MHC class II, B7.1, and/or the selectable neomycin resistance genes. Following limiting dilution cloning, several clones were chosen based on their surface expression of MHC class I, class II, and B7.1, as detected by indirect immunofluorescence staining (Fig. 3). All transfectants express similar levels of MHC class I as compared to parental 4T1 cells (Fig. 3, *a-h*). Two of the MHC class II transfectant clones (4T1/A^d-12 and 4T1/A^d-30) express similar levels of MHC class II, whereas the third class II transfectant (4T1/A^d-1) expresses higher levels (Fig. 3, *j-l*). Of the four B7.1 transfectants, two clones (4T1/B7.1-1 and 4T1/B7.1-6) express similar levels of B7.1, which are slightly higher than the levels expressed by the two other transfectants (4T1/B7.1-15 and 4T1/B7.1-23 (Fig. 3, *u-v*). 4T1 cells transfected with the empty parental vector (4T1/neo) do not express either MHC class II or B7.1 (data not shown), as observed with untransfected 4T1 cells (Fig. 3, *i* and *q*).

To test the immunogenicity and tumorigenicity of the class II and B7.1 transfectants, syngeneic female BALB/c mice were challenged in the abdominal mammary gland with 5×10^3 tumor cells, and the challenged mice were followed for primary tumor growth and metastasis formation. Fig. 4 shows the number of clonogenic tumor cells in the lungs *versus* TD at time of sacrifice (A-H), and the growth rate of the primary tumor (A-H, insets) for the various transfectants. With the exception of 4T1/A^d-30 (Fig. 4D, inset), all of the transfectants show some reduction in primary tumor growth rate and/or lack of tumorigenicity, although only the 4T1/A^d-12 transfectant does not form primary tumors in any of the inoculated mice (Fig. 4C). In contrast, the metastatic potential of both the class II⁺ and B7.1⁺ transfectants is markedly reduced relative to 4T1 cells. For example, 17 of 21 mice inoculated with class II⁺ transfectants contained <5,000 metastatic cells in the lung (Fig. 4, B-D), whereas 15 of 15 mice inoculated with wild-type 4T1 cells have 5,000-120,000 metastatic cells in the lung (Fig. 4A). For the B7.1⁺ transfectants, 19 of 20 inoculated mice contained 0-432 metastatic cells, with only one mouse displaying >10,000 tumor cells in the lungs (Fig. 4, E-H). Primary tumor growth in immunocompetent syngeneic mice, therefore, is inconsistently re-

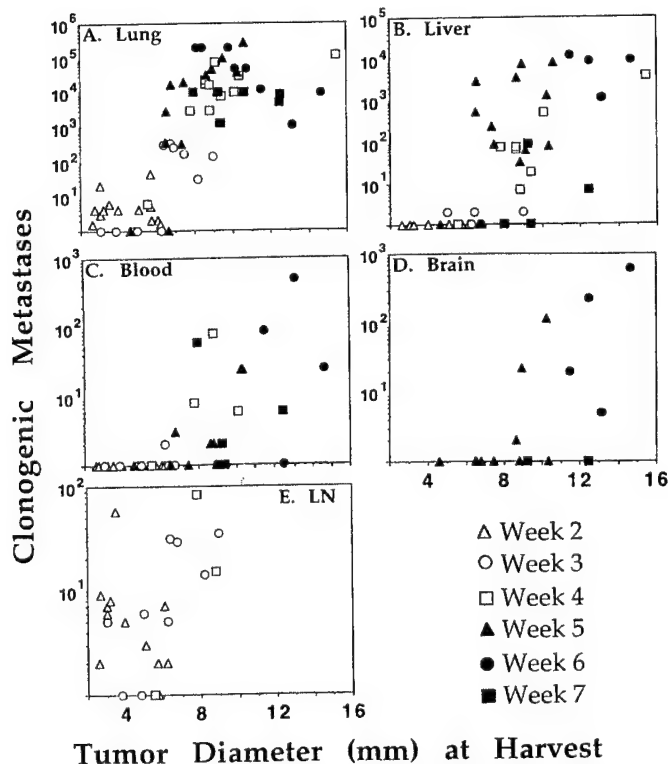


Fig. 2. 4T1 tumor cells spontaneously metastasize to the lungs (A), liver (B), blood (C), brain (D), and LN (E). Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Mice were sacrificed at varying times after inoculation (weeks 2-7), and the number of metastatic tumor cells was determined as described in "Materials and Methods." Data points, individual mice.

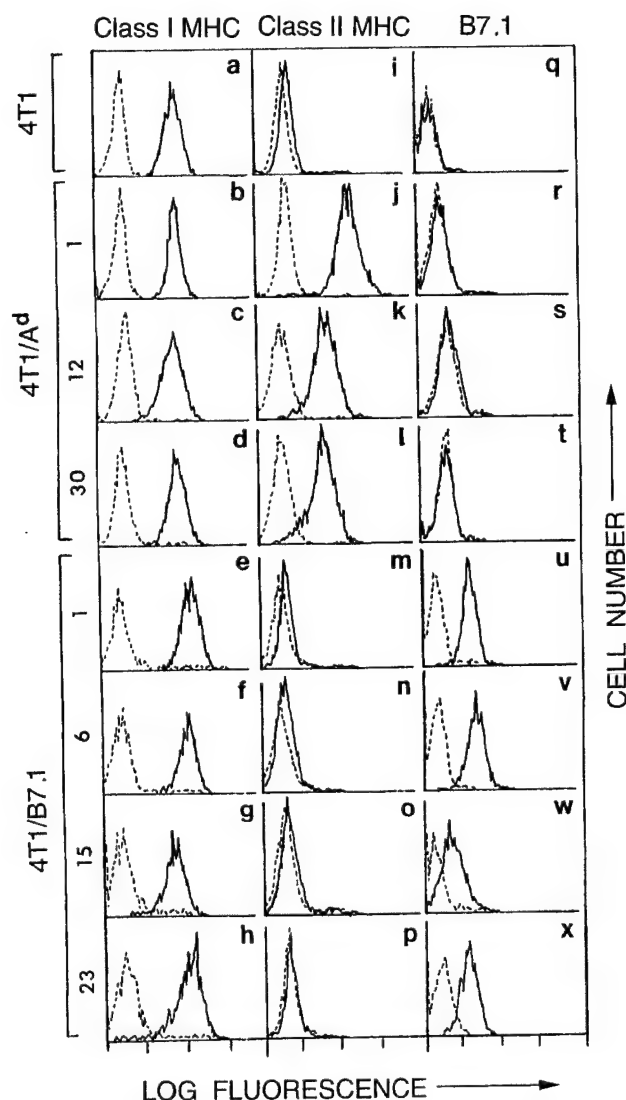


Fig. 3. 4T1 mammary carcinoma transfectants express either I-A^d class II MHC or B7.1 molecules. Parental 4T1 cells and transfectants were stained by indirect immunofluorescence as described in "Materials and Methods." Class I MHC expression (a-h) was measured using the mouse anti-H-2D^d mAb 34-5-8 (—) and irrelevant control mouse anti-H-2K^b mAb 16.3.1 (.....). Class II MHC expression (i-p) was measured using the mouse anti-A^d mAb MKD6 (—) and the isotype-matched irrelevant control mouse anti-A^{b,k} mAb 3JP (.....). B7.1 expression (q-x) was measured using the rat anti-B7.1 mAb 1G10 (—) with the conjugate alone (.....) as control. The X axis shows four logarithmic cycles of fluorescence intensity.

duced by expression of MHC class II or B7.1 genes; however, metastatic potential is reproducibly decreased.

Primary Tumor Growth and Metastasis Formation Are Regulated by T Lymphocytes. To determine whether T cell-mediated immunity is involved in the reduced tumorigenicity and metastatic spread of the class II⁺ and B7.1⁺ transfectants, T cell-deficient *nu/nu* mice were tumor-challenged (5×10^3 cells) and followed for primary tumor growth and metastasis formation. Two MHC class II transfectants and two B7.1 transfectants were used. As shown in Fig. 5, one of the class II⁺ transfectants (4T1/A^d-1; Fig. 5B) and one of the B7.1⁺ transfectants (4T1/B7.1-6; Fig. 5D) formed tumors and metastases in nude mice similar to unmodified wild-type 4T1 tumor cells (Fig. 5A). In contrast, 4T1/A^d-12 (Fig. 5C) and 4T1/B7.1-23 (Fig. 5E) lines formed primary tumor comparable to 4T1; however, their metastatic potential was much reduced relative to wild-type 4T1 tumor cells. To analyze the effects of T cells in immunocompetent *versus* T cell-deficient mice, primary tumor incidence in BALB/c

and BALB/c *nu/nu* mice were compared. As summarized in Table 3, 87% of the BALB/c *nu/nu* *versus* 20% of the BALB/c mice developed progressive primary tumor following s.c. challenge. The class II⁺ and B7.1⁺ transfectants, therefore, have different primary growth kinetics and metastasis formation in T cell-deficient nude mice *versus* immunocompetent BALB/c mice, suggesting that T lymphocytes are important effector cells for regulating tumor growth *in vivo*.

Immunization of Naive Mice with 4T1 Transfectants Expressing MHC Class II or B7.1 Protects against Metastatic Disease but not Primary Tumor Growth following Wild-type 4T1 Challenge.

The experiments of Figs. 1-5 suggest that the reduced primary tumor and metastasis formation of the class II⁺ and B7.1⁺ transfectants *versus* 4T1 cells is due to increased tumor cell immunogenicity. We, therefore, have tested the transfectants as immunotherapeutic agents. In the first regimen, naive, tumor-free syngeneic BALB/c mice were immunized i.p. with 10^6 irradiated transfectants and challenged s.c. 4 weeks later with 5×10^3 live 4T1 parental cells. Mice were sacrificed 5 weeks after the 4T1 challenge and clonogenic tumor cells measured in the lungs. As shown in Fig. 6, all of the transfectants provided some protection against 4T1 metastasis, with 4T1/A^d-12 and the mixture of 4T1/A^d-12 plus 4T1/B7.1-23 providing the maximum protection (<1400 clonogenic cells in each individual lung), and immunization with wild-type 4T1 providing minimal protection. Clonogenic metastatic cells in the liver and blood were also similarly reduced in the transfectant-treated animals (data not shown). Other organs were not monitored for metastatic cells. However, none of the transfectants significantly reduced the growth of the primary tumor (data not shown). Immunization of naive mice with the class II⁺ and/or B7.1⁺ transfectants significantly protects against spontaneous metastatic disease but does not affect primary tumor growth of wild-type 4T1 tumor.

Treatment of Tumor-bearing Mice with Transfectants Expressing MHC Class II or B7.1 Reduces Established Wild-type Metastatic Disease but Does Not Affect Primary Tumor Growth. To model a more realistic clinical situation and to test the transfectants more rigorously, the therapeutic efficacy of two transfectant clones was further tested in mice against established metastases. BALB/c mice were challenged s.c. with 5×10^3 wild-type 4T1 tumor cells and, starting at either day 9 or 14 after 4T1 challenge, they were given injections of irradiated transfectants (4T1/A^d-12 and/or 4T1/B7.1-6) twice a week until the day of sacrifice, approximately 4 weeks later. At the time of sacrifice, primary TDs of control-treated mice (i.e., mice given irradiated 4T1 cells), 6.8-12.5 mm, were comparable to TDs in transfectant-treated animals, 6.3-13.6 mm. The two-tailed *P* was 0.29 when tumor sizes of mice treated with control cells were compared with those of transfectant-treated mice combined. Lungs were subsequently removed, and the number of clonogenic tumor cells was determined. Because this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic cells in the lungs *versus* TD at the start of treatment. As shown in Fig. 7, administration of 4T1/A^d-12, 4T1/B7.1-6, or a mixture of cells significantly reduces the number of lung metastases (Fig. 7, B-D) relative to treatment with wild-type 4T1 cells (Fig. 7A) when primary TDs at the start of treatment were <4 mm. After transforming the number of clonogenic metastases to logarithmic values and analyzing as described in "Materials and Methods," the two-tailed *P* was 0.008 when control-treated mice were compared with transfectant-treated mice combined. When TDs, however, were >4 mm on the initial treatment day, no significant reduction in primary tumor growth or metastatic cells was seen (data not shown). Metastatic spread, therefore, can be significantly reduced by im-

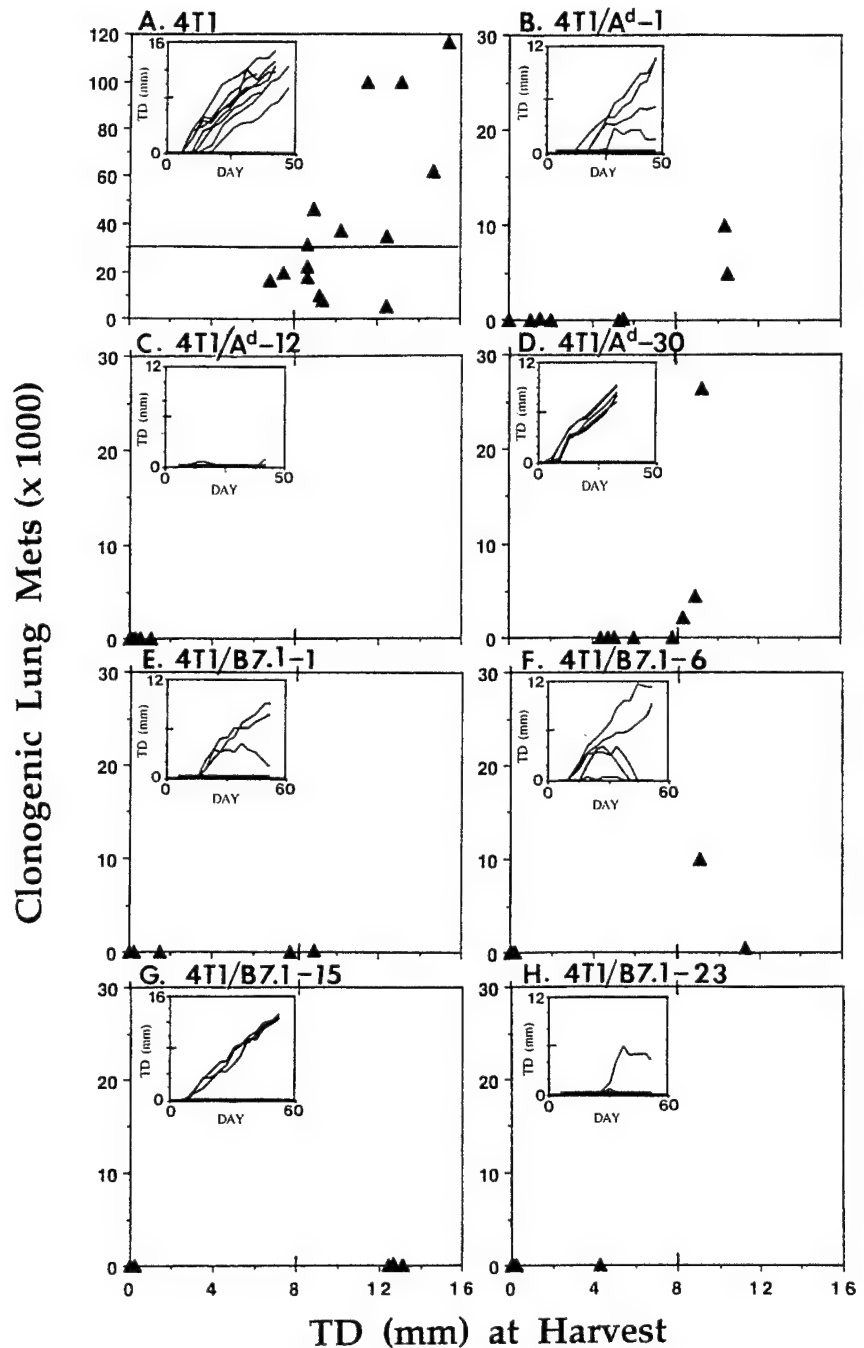


Fig. 4. Expression of either class II MHC or B7.1 reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (15 mice; A), 4T1/A^d-1 (9 mice; B), 4T1/A^d-12 (10 mice; C), 4T1/A^d-30 (8 mice; D), 4T1/B7.1-1 (5 mice; E), 4T1/B7.1-6 (5 mice; F), 4T1/B7.1-15 (5 mice; G), or 4T1/B7.1-23 (5 mice; H) and sacrificed 32–55 days later, and the number of metastatic cells in the lungs was determined as described in "Materials and Methods." Primary tumors were measured every 3–4 days. A–H, numbers of clonogenic lung metastases ($\times 1000$) versus the TD at the time the mice were sacrificed. \blacktriangle , individual mice. *Insets*, mean TD (Y axis) versus days postinoculation (X axis). *Lines*, individual mice. Note that the number of clonogenic lung metastases shown on the Y axis ranges from 0 to 120 in A, as opposed to a range of 0–30 for B–H.

munotherapy in mice carrying spontaneously metastatic established tumors, provided treatment originates when the primary tumor is <4 mm in diameter.

DISCUSSION

Many studies during the past 5–10 years have focused on developing immunotherapy strategies for the treatment of solid tumors and have used animal systems to model human disease and to test the efficacy of immunotherapy. Most of these studies have used transplanted primary solid tumors (6, 7) or short-term established experimental (i.v. induced) metastatic cancers, in which therapy was performed very early during metastatic disease (13–16). A small number of studies focused on spontaneous metastases; however, these models used severe combined immunodeficient mice or anatomically incor-

rect tumor challenge sites (17–19). In many cases, the growth characteristics and kinetics of the model tumors used did not closely follow the natural history of their corresponding human tumor and, hence, were not optimal model systems. In contrast to many mouse tumors, the BALB/c-derived 4T1 mammary tumor, originally derived by Miller and colleagues (29, 30) and others (28), shares many characteristics with its human counterpart mammary carcinoma. For example, 4T1 spontaneously metastasizes while the primary tumor is in place, analogous to human mammary tumors. Sites of metastasis are common between the mouse and human malignancies: spreading first to the lungs and liver in 24–77% and 22–62% of women, respectively, *versus* $>95\%$ and $>75\%$, respectively, of BALB/c mice (Table 2; Refs. 38–41). Metastasis to the central nervous system is characteristically less frequent than metastasis to other sites in both

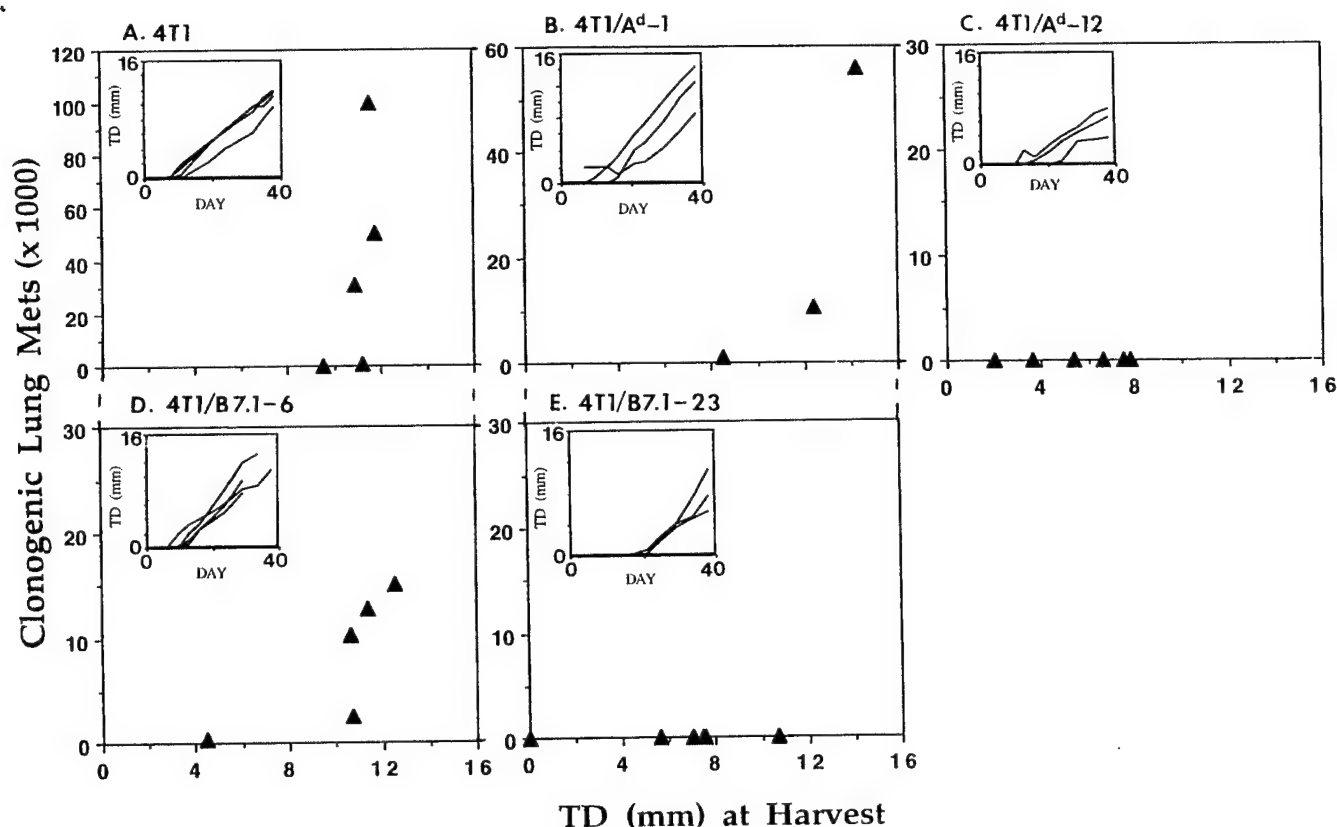


Fig. 5. Different immune effector cells alter primary tumor growth versus spontaneous metastasis formation. BALB/c *nu/nu* mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (5 mice; A), 4T1/A^d-1 (3 mice; B), 4T1/A^d-12 (6 mice; C), 4T1/B7.1-6 (5 mice; D), or 4T1/B7.1-23 (6 mice; E), and tumor growth was measured every 3–4 days. Data are plotted as in Fig. 4. Note that the number of clonogenic lung metastases shown on the Y axis ranges from 0 to 120 in A, as opposed to ranges of 0–60 for B and 0–30 for C–E.

Table 3. Tumor incidence of 4T1 transfectants in syngeneic BALB/c versus BALB/c *nu/nu* mice

Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 transfectant 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Tumor challenge	Tumor incidence	
	BALB/c	BALB/c <i>nu/nu</i>
4T1/A ^d -1	3/10	3/3
4T1/A ^d -12	1/10	5/6
4T1/B7.1-6	2/5	7/8
4T1/B7.1-23	1/5	5/6

humans and mice (30% and 40%, respectively) and, statistically, occurs later in the disease process (Table 2; Refs. 41 and 42).

In addition to its growth characteristics, the 4T1 tumor has several experimental characteristics that make it an ideal model for testing immunotherapy strategies. A major asset is its stable resistance to 6-thioguanine, enabling the precise quantitation of very small numbers of tumor cells, long before they could be detected visually or accurately quantitated by other methods. Because metastasis to the lungs precedes and always accompanies metastasis to other organs (Table 2), quantitation of lung metastases accurately assesses metastatic disease. The similarity in growth between the 4T1 tumor and human mammary cancer plus the ease of assessing metastatic disease, therefore, make the mouse 4T1 tumor an excellent model for testing potential immunotherapy strategies.

Previous immunotherapy studies using MHC class II and/or B7.1-expressing tumor cells as cell-based vaccines have dealt predominantly with solid, primary tumors (7–9). Here, these vaccines are used

for the treatment of metastatic disease. The current studies are also distinct from earlier studies using a variety of cell-based vaccines, including cytokine-transduced/transfected tumor cells, in that spontaneous, established metastases are being treated, rather than short-term experimental (i.v.) metastases. These disease conditions much more closely mimic those of human breast cancer patients, and hence, the

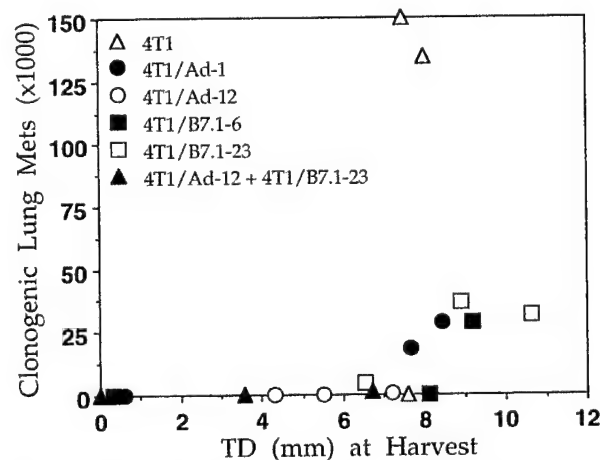


Fig. 6. Immunization with MHC class II⁺ or B7.1⁺ transfectants protects naive mice against metastatic disease from parental 4T1 tumor challenge. Syngeneic BALB/c mice (three mice/group) were vaccinated i.p. with 1×10^6 irradiated parental 4T1 cells (Δ), 4T1/A^d-1 (\bullet), 4T1/A^d-12 (\circ), 4T1/B7.1-6 (\blacksquare), 4T1/B7.1-23 (\square), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-23 (\blacktriangle). Four weeks later, mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. Five weeks postparental tumor challenge, the TD and the number of clonogenic lung metastases were measured.

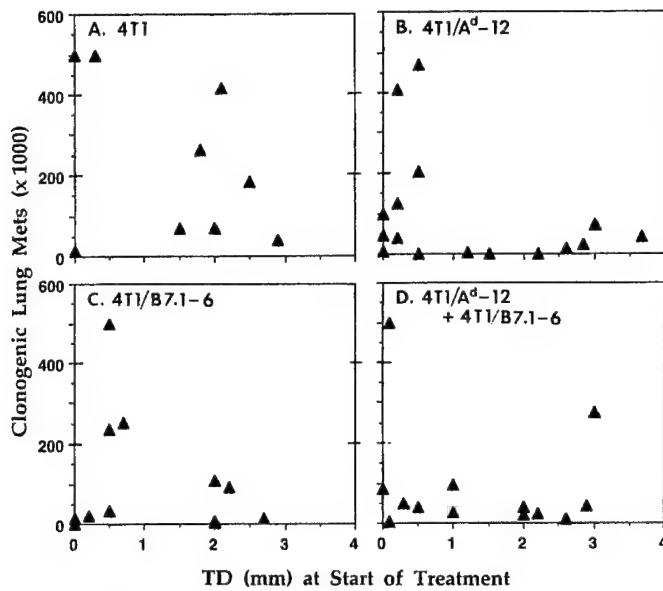


Fig. 7. Immunotherapy of established 4T1 tumors with MHC class II⁺ and/or B7.1⁺ transfectants reduces metastatic disease. Syngeneic BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. At day 9 or 14 postparental tumor challenge, the TD was measured, and the therapeutic injections began. Mice were treated i.p. twice a week until the time of sacrifice with 1×10^6 irradiated parental 4T1 (A), 4T1/A^d-12 (B), 4T1/B7.1-6 (C), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-6 (D) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge, and the number of clonogenic lung metastases was determined. The data are plotted as the TD at the time the therapeutic treatment began versus the number of clonogenic lung metastases ($\times 1000$) at the time of sacrifice. \blacktriangle , individual mice. Statistical analysis was performed using a Student's *t* test for unequal variances as described in the text (two-tailed $P = 0.008$).

observed results may be useful in projecting experimental animal results to human clinical situations.

Treatment of mice carrying 9–14-day established 4T1 tumors with MHC class II and/or B7.1-transfected tumor cells results in a dramatic reduction in the number of metastatic tumor cells relative to mice treated with wild-type 4T1 (Fig. 7), suggesting that such cell-based vaccines may be useful immunotherapeutic agents for the treatment of metastases. The finding that metastatic growth is greatly reduced or eliminated, whereas primary tumor growth is not significantly impacted, is surprising and suggests that immunotherapy may be more useful against metastatic disease than against primary tumor. Because many primary tumors can be successfully surgically resected whereas many metastatic lesions are refractile to current therapy, immunotherapy may have a unique role in cancer treatment.

Because mice with primary tumors with TDs of >2 mm contain LN and lung metastatic cells (Fig. 2), the immunotherapy is limiting proliferation of pre-established metastases. Likewise, because treatment of naive mice produces some animals with no metastases, the immunotherapy is also preventing establishment of new metastases. Therefore, although not routinely curative, this immunotherapy may slow progression of metastatic disease.

Previous therapy studies with B7.1 transfected tumors and primary or experimental metastases indicated that costimulatory molecule expression was effective in vaccines containing “moderately” immunogenic tumor cells but not in vaccines containing “poorly” immunogenic tumor cells (7). By definition, 4T1 cells are poorly immunogenic because immunization of tumor-free mice with irradiated wild-type cells does not provide protective immunity against subsequent challenge with wild-type tumor cells (Figs. 6 and 7). Because immunization with B7.1 transfected tumor cells does not result in reduced primary tumor growth in the immunotherapy protocol, our results agree with these earlier studies (7). However, the finding that B7.1-

transfected tumor cells promote significantly reduced metastatic growth in the therapy protocol (Fig. 7) revives B7.1 as a potential candidate for immunotherapy.

The mechanism by which the class II⁺ and B7.1⁺ transfectants are providing their protection is not clear. Because these transfectants displayed varying *in vivo* phenotypes, different types of effector cells may be activated. In most cases, T cells were important in regulating primary tumor growth (Fig. 5); however, their role in outgrowth of metastases is less clear cut. This could easily be explained by an enhancement of nonspecific effectors, such as lymphokine-activated killer cells and/or NK cells, as it has been previously shown that B7.1 can induce NK activity against tumors (32, 43). Alternatively, limiting dilution cloning of the transfectants may have cloned out tumor cells that lost their ability to metastasize (44). Regardless of the *in vitro* and *in vivo* phenotypes of the transfectants (*i.e.*, level of expression of class II and/or B7.1, metastatic potential, and tumorigenicity in BALB/c versus *nu/nu* mice), most clones provide some protection against wild-type metastatic disease (Figs. 6 and 7). Thus, these studies suggest that most transfectants will be useful as vaccines and that cell-based vaccines may be more effective than previously thought.

Transfection of tumor cells with MHC class II plus B7.1 genes was originally designed to produce tumor cells that could directly present antigen to CD4⁺ T_H cells and CD8⁺ CTL and, thereby, facilitate optimal antitumor immunity (9, 45). Genetic experiments using bone marrow chimeras and sarcoma tumor cells support this hypothesized mechanism of CD4⁺ T-cell activation and demonstrate that the genetically modified tumor cells function as the APC for tumor-encoded antigen (46, 47). In contrast, class I-restricted tumor-encoded antigen appear to be presented indirectly via host-derived APCs (48–50). Increased antitumor activity following immunization, therefore, is probably the result of enhanced presentation of tumor antigens and the subsequent activation of multiple helper and effector cell populations.

Why the effectiveness of this treatment is limited to mice with starting tumors with TDs of <4 mm is unclear. Factors such as immunosuppression of tumor-bearing individuals, immunogenicity of tumor antigens, the timing of the developing immune response versus outgrowth of the tumor, and involvement of nonspecific effector cell types (*i.e.*, lymphokine-activated killer cells, NK cells, and macrophages) have been discussed at length in the context of other immunotherapy approaches (51–53), and some or all of these factors may be implicated here. Optimal T-cell activation is achieved when B7.1 and MHC class II molecules are expressed by the same APC (9, 54). Our cell-based vaccine, therefore, might be more effective if double transfectants were used rather than the mixture of single transfectants tested in this study. Regardless of the limitations, however, the promising therapeutic responses are encouraging for further testing and development of this approach either alone or in combination with other immunotherapeutic and/or conventional modalities.

ACKNOWLEDGMENTS

We thank Drs. T. Armstrong and S. Baskar for their critical review of this manuscript, V. Gunther for her help with dissections, S. Mason for animal care, T. Iamonte and Dr. B. Bradley for their assistance with statistical analysis, and F. Baldwin for her graphic expertise.

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Cooperativity of *Staphylococcal aureus* Enterotoxin B Superantigen, Major Histocompatibility Complex Class II, and CD80 for Immunotherapy of Advanced Spontaneous Metastases in a Clinically Relevant Postoperative Mouse Breast Cancer Model¹

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ABSTRACT

One of the leading causes of death for women is metastatic breast cancer. Because most animal tumors do not accurately model clinical metastatic disease, the development of effective therapies has progressed slowly. In this study, we establish the poorly immunogenic mouse 4T1 mammary carcinoma as a postsurgical animal model. 4T1 growth characteristics parallel highly invasive human metastatic mammary carcinoma and, at the time of surgery, the extent of disease is comparable with human stage IV breast cancer. Progress in understanding the immune response has led to innovative immune-based anticancer therapies. Here, we test in this postsurgical model, a novel cell-based vaccine, combining MHC class II, CD80 (B7.1), and SEB superantigen. Effective treatment of tumor-bearing mice with this immunotherapy requires expression of all three molecules. Mean survival time is extended from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Increased survival is accompanied by a maximum of 100-fold decrease in clonogenic lung metastases. These therapeutic effects are particularly noteworthy because: (a) the postoperative model demonstrates that early metastases responsible for morbidity are established by 2 weeks after tumor inoculation with 7×10^3 parental 4T1 cells into the mammary gland; (b) the immunotherapy is started 4 weeks after tumor inoculation when the mice contain extensive, pre-established, disseminated metastases; and (c) CD4⁺ and CD8⁺ T cells are required for the effect.

INTRODUCTION

As a result of recent discoveries and advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1, 2). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, few immunotherapeutic approaches are targeting disseminated metastatic disease, for which conventional therapies frequently have limited success. Development of therapies for the treatment of metastatic disease is complicated by the shortage of animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma model. The 4T1 tumor shares many characteristics with its human counterpart (3), making it an excellent animal model.

In most clinical situations, primary mammary tumors are cured by surgery, yet approximately 33% of women successfully treated for primary tumors die subsequently from spontaneous metastatic disease

(4). To further refine the 4T1 system and to more closely parallel clinical disease, we have now developed a postsurgical model of the 4T1 mammary tumor. In this model, mice receive inoculations s.c. in the abdominal mammary gland and the primary tumor is allowed to grow progressively, become extensively vascularized, and metastasize. The primary tumor is then surgically resected, and therapy with the cell-based vaccines is initiated.

The cell-based vaccines consist of tumor cells transfected with syngeneic MHC class II (I-A^d) and CD80 (B7.1) costimulatory molecule genes and were designed to enhance activation of tumor-specific CD4⁺ T lymphocytes via improved presentation of tumor-encoded class II-restricted epitopes. Although CD8⁺ T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that CD4⁺ T lymphocytes also play a critical role in effective antitumor immunity (5–9). Whereas our previous vaccines showed significant reduction of established, spontaneous metastatic tumor, the antitumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (3). In addition, we did not assess the effects of immunotherapy on survival. Furthermore, the vaccine was tested in mice with metastatic disease and carrying intact primary tumor so the model did not mimic the clinical situation in which primary tumor would have been surgically removed before initiation of immunotherapy. We now report a second-generation cell-based vaccine that is significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer and that is tested in a postsurgical model. The new vaccine incorporates a gene encoding the bacterial toxin SEB³. SEB is a sAg that when complexed with MHC class II molecules on APCs is a potent polyclonal activator of CD4⁺ T lymphocytes (10, 11). Although CD4⁺ T-cell activation by SEB is not antigen specific, we reasoned that the addition of SEB to the MHC class II/CD80 vaccine will provide additional activation signals to the CD4⁺ T cells that have been activated in an antigen-specific fashion by the MHC class II⁺ CD80⁺ vaccinating cells.

MATERIALS AND METHODS

cDNA Expression Vectors. The expression vectors pH β -Apr-1-neo containing MHC class II (I-A^d, I-A^β) and mouse B7.1 have been described previously (3). The SEB gene (12) was subcloned into the *SalI/BamHI* site of the pH β -Apr-1-neo expression vector. The final construct, pH β -SEB-neo, contains the amino acid sequence for the mature SEB protein minus the signal peptide and confers resistance to G-418. The pZeoSV2 plasmid was purchased from Invitrogen (San Diego, CA).

Animals, Cell Lines, and Transfectants. Female BALB/c and BALB/c *nu/nu* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and used at 8 weeks of age. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma (13), was kindly supplied

Received 11/15/99; accepted 3/17/00.

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¹Supported by grants from the U.S. Army Research and Development Command (DAMD17-94-J-4323) and the NIH (RO1 CA52527). B. P. is supported by a postdoctoral fellowship from the U.S. Army Research and Development Command (DAMD17-97-1-7152).

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³The abbreviations used are: SEB, *Staphylococcal aureus* enterotoxin B; sAg, superantigen; APC, antigen-presenting cell; TD, mean tumor diameter.

by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI) and grown in culture as described previously (3). Transfectants were made to express MHC class II and CD80, or SEB by using lipofectin (Life Technologies, Inc.) according to manufacturer's instructions. Cells were selected with 400 $\mu\text{g}/\text{ml}$ G-418 (Life Technologies, Inc.) or 200 $\mu\text{g}/\text{ml}$ zeocin (Invitrogen), cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry, as described previously (3).

SEB Assay. Naive BALB/c spleen cells ($5\text{--}10 \times 10^5$) were cultured in serial dilutions of transfectants' supernatants or purified SEB (Sigma Chemical Co., St. Louis, MO), as indicated. To demonstrate specific SEB activity, a polyclonal rabbit antibody against SEB (Sigma Chemical Co.) was added to cultures, as indicated. After 3 days in culture, spleen cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, as described previously (14).

Surgery. All surgical supplies and surgical equipment were purchased from Henry Schein Veterinary & Medical Supply Catalogue (Melville, NY) and Roboz (Rockville, MD), respectively, unless indicated otherwise. Before surgery, animals were weighed and anesthetized with i.p. injections (0.02 ml/g body weight) of 2.5% recrystallized avertin (2,2,2-tribromomethanol; Sigma Chemical Co.). Once the animals were unconscious, the tumor-bearing abdominal area was prepared and sterilized for surgery by shaving with Oster Finisher Trimmer, followed by 2 washes each with diluted Nolvasan surgical scrub (chlorhexidine) and isopropanol. Tumors were resected with sterilized surgical instruments, removing the smallest amount of skin tissue as possible. Wounds were closed with either Nexaband liquid or stainless steel 9-mm wound clamps with a Mikon autoclamp applier, as necessary. Wound clamps were removed 10 days after surgery with a Mikon autoclamp remover. Mice were monitored for survival, and those that died from surgery (within 1–4 days after surgery, survival rate of 67–80%) were not included in the experiment. All mice were autopsied at the time of death to confirm the presence of lung metastases as well as recurrence of the primary tumor.

Tumor Challenges, Metastases Assays, and *in Vivo* Depletions. Mice were challenged s.c. in the abdominal mammary gland with ($7 \times 10^3/50 \mu\text{l}$) parental 4T1 tumor cells. Primary tumor growth and spontaneous metastases were measured as described previously (3). Depletions of CD4⁺ and CD8⁺ T cells were performed as described previously (15). Splenocytes of all depleted mice were checked by immunofluorescence for depletion at the conclusion of the experiment. Mice depleted for CD4⁺ or CD8⁺ T cells had <4% or 7% of CD4⁺ T cells or CD8⁺ T cells, respectively.

Statistical Analyses. To determine the statistical significance of the data, the Tukey's Honestly Significant Difference Test was performed at a *P* set at 0.05. The Tukey's test is a multicomparison test that determines the statistical significance of data sets of size 3 or greater and allows for unequal sample size (*n*) and sample variances (16). To determine the statistical significance of the effects of immunotherapy on primary tumor growth, the Student's *t* test for unequal variances (Microsoft Excel, version 5.0) was performed.

RESULTS

Tumor Lethality Is Due to Early Metastases. Our previous studies have demonstrated that the BALB/c-derived 4T1 mammary carcinoma is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes throughout the body in a pattern similar to human breast cancer (3). For example, primary 4T1 tumors that have been established for 2–3 weeks in BALB/c mice typically metastasize to the lymph nodes, lungs, and livers in 86%, 79%, and 20% of mice, respectively, and the numbers of micrometastatic cells found in these organs range between 2–57, 1–338, and 0–1, respectively. In addition, as the primary tumors age (*i.e.*, by 4–5 weeks), the incidence of metastases in the lungs, livers, and now brains increases to 91%, 82%, and 36% of mice, respectively, and the range of metastatic cells for these organs is between 6–250,000, 7–7800, and 1–116, respectively (3).

As shown in Fig. 1, 4T1 is also similar to human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early (week 2) during primary tumor growth. Groups of female

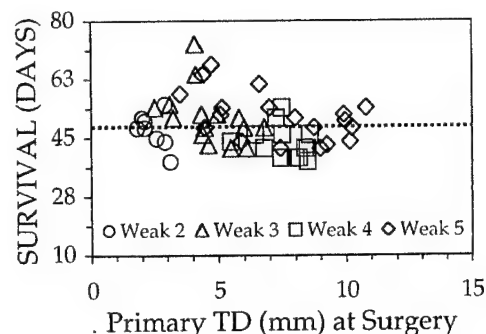


Fig. 1. Early spontaneous metastases are responsible for mortality. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. Primary TDs were measured, and tumors were surgically resected at varying times after inoculation (weeks 2–5). Each point represents the survival time in days after primary tumor challenge for an individual mouse.

BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 wild-type 4T1 tumor cells. Starting at 2 weeks after challenge and continuing at 1-week intervals, TDs were measured, primary tumors were surgically removed, and mice were followed for survival times. Because we were interested in knowing whether primary tumor size affected survival time, the data have been plotted as tumor size (mm) at the time of surgery *versus* the number of days the mice survived after 4T1 tumor challenge. As shown in Fig. 1, the average survival time of 55 of 58 mice was $48.9 (\pm 7.4)$ days, whereas the remaining 3 mice, whose tumors were <3 mm in diameter at the time of surgery, lived >90 days and did not die of metastatic cancer. Surprisingly, all mice that died from spontaneous metastatic disease showed approximately the same mean survival time regardless of the size of the primary tumor at the time of surgery. These results demonstrate that lethal metastasis is established as early as 2 weeks after inoculation of primary tumor, that the mean survival time is 7 weeks, and that surgical removal of primary tumor does not change these kinetics.

The surgical experiments of Fig. 1, combined with our previous studies (3), demonstrate that the 4T1 system is comparable with human stage IV breast cancer. Human breast cancer at stage IV is characterized by several diagnostic factors: (a) the presence of edema and ulcerations of the skin in and around the tumor burden; (b) extension of the primary tumor to the chest cavity lining; (c) presence of metastatic cells in the draining lymph nodes; and (d) presence of metastases in distant organs (4). This postsurgical 4T1 system exhibits all of these characteristics. Metastases are present in the draining lymph nodes and distant organs as early as week 2 and progress into more advanced metastatic disease with time (3). All of the resected 4T1 tumors, regardless of their size at the time of surgery, were highly vascularized. The primary tumors displayed edema when there was a TD ≥ 4 mm and ulcerations of the skin in approximately 70% of tumors regardless of size (data not shown). Most tumors extended to the lining of the peritoneal cavity, whereas invasion through the peritoneal lining was less frequent (<5%) and only occurred when primary TD was large (5–6 mm; data not shown). Therefore, at the time of surgery, the mouse 4T1 tumors are comparable with stage IV human breast cancer and are a much more rigorous animal model for the development of effective therapies than other experimental systems reported in the literature.

Postoperative Treatment of Mice with Transfectants Expressing MHC Class II, CD80, and SEB Increases Survival. Previously, we have shown that therapy with transfectants expressing MHC class II or CD80 reduced metastatic disease in a model where the primary 4T1 tumor remained *in situ* and had been established for 9–14 days.

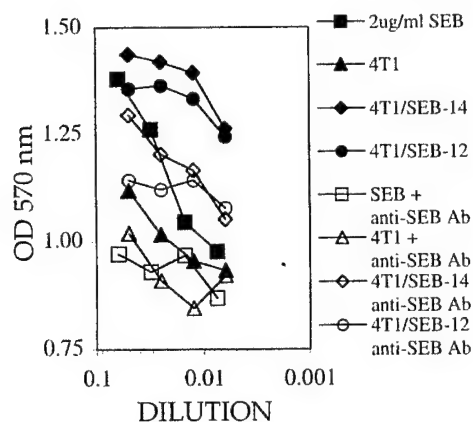


Fig. 2. Supernatants from 4T1/SEB transfectants stimulate proliferation of spleen cells. Naïve BALB/c splenocytes were cocultured with soluble SEB starting at a concentration of 2 µg/ml (■) or supernatants from parental 4T1 (▲, △), 4T1/SEB-12 (●, ○), or 4T1/SEB-14 (◆, ◇) transfectants in the presence (open symbols) or absence (filled symbols) of antibody specific for SEB.

The success of this treatment, however, was limited to small tumor burdens and did not completely eliminate spontaneous metastases (3). A potential problem with this earlier therapy is that the transfectants did not coexpress MHC class II and CD80, and previous data using a mouse sarcoma showed that coexpression of these molecules is synergistic (15). We have, therefore, generated 4T1 transfectants that coexpress MHC class II and CD80 as detected by indirect immunofluorescence staining (data not shown) to test this hypothesis. To further increase the potency of the vaccine, we have combined the MHC class II⁺/CD80⁺ double transfectants (4T1/A^d/B7.1) with SEB⁺ transfectants (4T1/SEB), reasoning that SEB may provide additional proliferation signals to the tumor-specific T cells activated via the MHC class II/CD80 interaction.

SEB expression was tested by coculturing supernatants of transfectants with naïve BALB/c spleen cells and monitoring lymphocyte proliferation. To determine a relative amount of SEB secretion, splenocytes were also cultured with soluble SEB. As shown in Fig. 2, supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) stimulated splenocyte proliferation as efficiently as soluble SEB at a concentration of 2 µg/ml. This activity was reduced on the addition of a polyclonal anti-SEB antibody, demonstrating that the spleen cell proliferation was due to SEB expression by the 4T1 transfectants. Supernatants from parental 4T1 cells as well as supernatants from 4T1 cells transfected with empty vector (4T1/neo) did not induce proliferative responses (Fig. 2 and data not shown). Therefore, the 4T1/SEB transfectants secrete SEB, which induces splenocyte proliferation comparable with proliferation induced by soluble exogenously added SEB.

Vaccines such as the 4T1 transfectants are likely to be most useful for the treatment of disseminated spontaneous metastatic disease because primary tumors usually can be eliminated by surgery. Therefore, we have tested the combination vaccine in mice with established, disseminated spontaneous metastases following surgical removal of the primary tumor. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 wild-type 4T1 tumor cells. The tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. The experiments in Fig. 1 and our previously published results (3) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery (3 weeks after primary tumor challenge), the primary TD in each treatment group ranged between 3.5 mm and 5.7 mm. The extent of spontaneous metastatic cancer at this time is significantly more

advanced than that tested in other immunotherapy experiments. Therapeutic injections of irradiated vaccine cells (1×10^6 total) were started 1 week after surgery (i.e., 4 weeks after initial tumor challenge) and were administered every 3–5 days for the duration of the experiment. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results in Fig. 3 are plotted as the survival time *versus* the size of the primary tumor at the time of surgery. A line denoting the average survival time of the 4T1-treated control group (45 days) is included to demonstrate the effects of the vaccine on survival. The survival time in days for 4T1-treated (Fig. 3A), 4T1/SEB-treated (Fig. 3B), 4T1/A^d/B7.1-treated (Fig. 3C), and 4T1/SEB+4T1/A^d/B7.1-treated (Fig. 3D) animals was 35–52, 40–59, 47–54, and 41–74 days, respectively. Statistical analyses using the Tukey's Honestly Significant Difference Test revealed that only the treatment with a 1:1 mixture of 4T1/A^d/B7.1+4T1/SEB cells significantly increases the survival time of mice with established wild-type metastatic disease ($P = 0.05$). Treatment with either 4T1/SEB alone or 4T1/A^d/B7.1 alone does not significantly increase survival. Therefore, therapy with this cell-based vaccine requires expression of all three molecules to extend mean survival time from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Although this increase in survival time is relatively small, it is statistically significant and compelling because the immunotherapy was started at week 4 and untreated and/or 4T1-treated mice begin to die as early as 5 weeks after tumor challenge.

Increase in Survival Correlates with Reduction of Metastatic Cancer. To demonstrate that the increase in survival was due to a reduction of spontaneous metastatic cancer, lungs from therapy-treated animals were harvested and the number of clonogenic metastases was quantitated as described previously (3). Female BALB/c mice were challenged s.c. in the abdominal mammary gland with

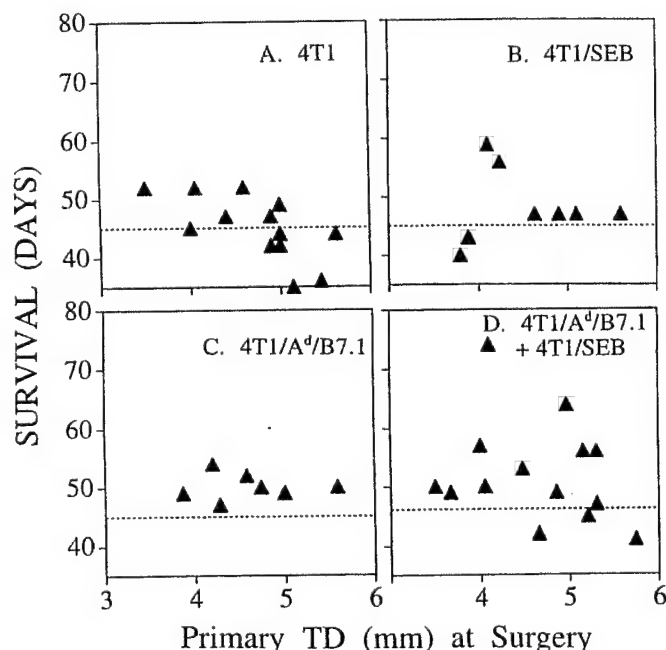


Fig. 3. Immunotherapy of established wild-type spontaneous metastases with a mixture of MHC class II/CD80 and SEB transfectants increases survival. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. Primary tumors were measured and surgically resected 21 days after parental tumor challenge. Mice were treated every 3–5 days starting at day 28 with i.p. injections of 1×10^6 total cells of irradiated parental 4T1 (13 mice; A), 4T1/SEB (8 mice; B), 4T1/A^d/B7.1 (7 mice; C), or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB cells (14 mice; D). The 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB therapy group is significantly different from the 4T1 control group ($P = 0.05$, Tukey's Honestly Significant Difference Test).

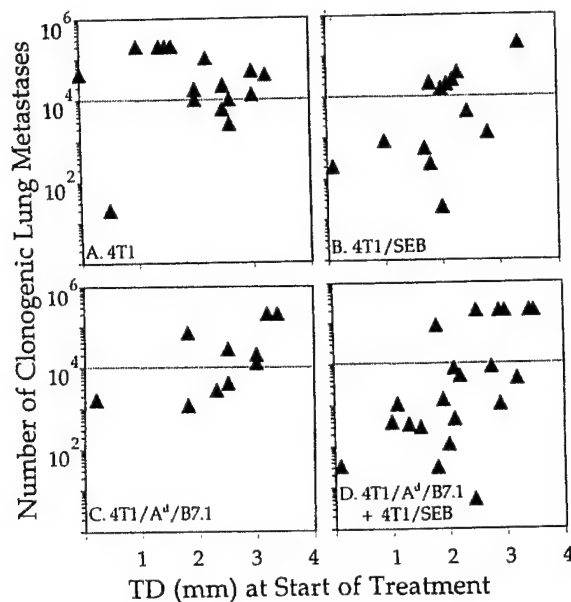


Fig. 4. Immunotherapy of established 4T1 tumors with MHC class II/CD80 and/or SEB transfectants reduces metastatic disease. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. At 14 days after parental tumor challenge, the TDs were measured and the therapeutic injections started. Mice were treated i.p. twice a week until the time of sacrifice with 1×10^6 total cells/injection of irradiated parental 4T1 (A), 4T1/SEB (B), 4T1/A^d/B7.1 (C), or a 1:1 mix of 4T1/A^d/B7.1 plus 4T1/SEB (D) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge, and the number of clonogenic lung metastases was determined. Each triangle represents an individual mouse. The 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB therapy group is significantly different from the 4T1 control group ($P = 0.05$, Tukey's Honestly Significant Difference Test).

7×10^3 wild-type 4T1 tumor cells. Starting 2 weeks after challenge, they were given i.p. injections of irradiated vaccine cells (1×10^6 total/injection) twice a week until the day of sacrifice. At the time of sacrifice (6 weeks after the initial primary tumor challenge), primary TD of control-treated mice (i.e., mice given irradiated 4T1 cells) were comparable with TD of transfectant-treated animals (6.5–10.5 mm and 6.2–11.2 mm, respectively; two-tailed $P = 0.61$). Therapy with the transfectants, therefore, does not reduce primary tumor growth, which concurs with our earlier studies using MHC class II or CD80 vaccines alone (3).

To assess the metastatic disease, lungs from the treated mice were removed, dissociated into single cell suspensions, and plated in culture medium containing 6-thioguanine to determine the number of clonogenic tumor cells. Ten days later, the number of clonogenic metastatic cells could be enumerated because 4T1 cells are resistant to 6-thioguanine, whereas normal cells are not resistant and die. As in Fig. 3, we are interested in determining whether primary tumor size effects vaccine efficacy, therefore, the results are plotted as number of clonogenic metastatic cells in the lungs versus TD at the start of treatment. A line denoting a level of 10,000 tumor cells in the lungs is also included because >85% of untreated tumor-bearing mice contain >10,000 metastatic cells in their lungs after 42 days of primary tumor growth (3). As shown in Fig. 4, administration of the 4T1 transfectants significantly reduces the number of lung metastases (Fig. 4, B–D) relative to treatment with wild-type 4T1 cells (Fig. 4A). For example, 13 of 16 (81.2%) mice treated with irradiated parental cells (Fig. 4A) contained >10⁴ clonogenic lung metastases, which contrasts 12 of 23 (52.2%) mice treated with either 4T1/SEB or 4T1/A^d/B7.1 (Fig. 4, B and C) and 6 of 21 (28.6%) mice treated with a mixture of transfectants (Fig. 4D). After transforming the number of clonogenic metastases to logarithmic values and analyzing these data using the Tukey's Honestly Significant Difference Test, we found that

only treatment with a 1:1 mixture of 4T1/SEB+4T1/A^d/B7.1 cells (Fig. 4D) significantly reduced the number of clonogenic lung metastases ($P = 0.05$). Treatment with either 4T1/SEB alone (Fig. 4B) or 4T1/A^d/B7.1 alone (Fig. 4C) did not significantly decrease the number of clonogenic lung metastases. Previously, we demonstrated that therapy with MHC class II⁺/CD80⁺ vaccines statistically significantly reduced clonogenic lung metastases in 50% of mice whose immunotherapy was initiated 9–14 days after tumor challenge but this reduction corresponded to only a 10-fold maximum reduction when compared with the control group (3). In contrast, treatment of mice carrying 14-day established primary and metastatic tumor with the combination therapy of tumor cell transfectants expressing MHC class II, CD80, and SEB genes decreases spontaneous metastases in the lung by a maximum of 100-fold. Therefore, effective immunotherapeutic treatment of tumor-bearing mice with extensively established spontaneous metastases requires expression of all three molecules.

Reduction of Established Wild-Type Metastases with MHC class II, CD80, and SEB Immunotherapy Requires Both CD4⁺ and CD8⁺ T cells. The concept of combining SEB with MHC class II and CD80 was based on the hypothesis that SEB is a potent polyclonal activator of CD4⁺ T lymphocytes (10, 11) and would provide additional activation signals to CD4⁺ T cells that have been activated in an antigen-specific fashion by the MHC class II⁺ CD80⁺ vaccinating cells. Therefore, we tested the immunotherapy described in Fig. 4 in CD4- or CD8-depleted animals and BALB/c *nu/nu* mice. As shown in Fig. 5, C and D, depletion of CD4⁺ or CD8⁺ T cells (monoclonal antibodies GK1.5 and 2.43, respectively) eliminates the therapeutic effect of the MHC class II⁺, CD80⁺, SEB⁺ vaccine against spontaneous metastases, whereas depletion with control ascites (Fig. 5B) has no effect. In addition, the combination vaccine does not reduce metastatic disease in BALB/c *nu/nu* mice (Fig. 5F). Collectively, these data demonstrate that the three transfectant genes of the cell-based vaccines are working cooperatively to optimally activate both CD4⁺ and CD8⁺ T lymphocytes and that these lymphocyte populations are essential for the therapeutic effect.

DISCUSSION

sAgs, including SEB, have been previously recognized as potential reagents for up-regulating T lymphocyte responses against tumors. However, their use has been limited and they have not been combined with other factors that might optimize their therapeutic efficacy. For example, several studies describe redirected T-cell activation using sAgs coupled to tumor-specific monoclonal, anti-idiotypic, or bifunctional antibodies (17–19). SEB has also been administered systemically along with tumor cells, and SEB DNA has been inoculated intratumorally along with cytokine DNA to reduce primary tumor growth (20, 21). In addition, sAgs have been used to activate tumor-draining lymph node T cells *ex vivo* for adoptive transfer into tumor-bearing animals (22, 23). All of these approaches produce some reduction in primary tumor growth and/or decrease in metastatic lesions. However, the test settings have involved relatively small primary tumor and/or very small metastatic tumor burdens, which do not mimic the clinical situation. These results, taken together with the SEB transfectant tumor vaccines presented in this study, show that SEB expression alone has only a modest effect on metastatic tumor progression. However, as shown in this study, the antitumor effect of SEB on highly advanced spontaneous metastases is more effective when combined with the cell-based vaccine containing MHC class II and CD80 molecules.

The modified tumor cells may function directly as APCs for the initial activation of tumor-specific CD8⁺ and CD4⁺ T cells following immunization. Previous studies demonstrate that both CD8⁺ and

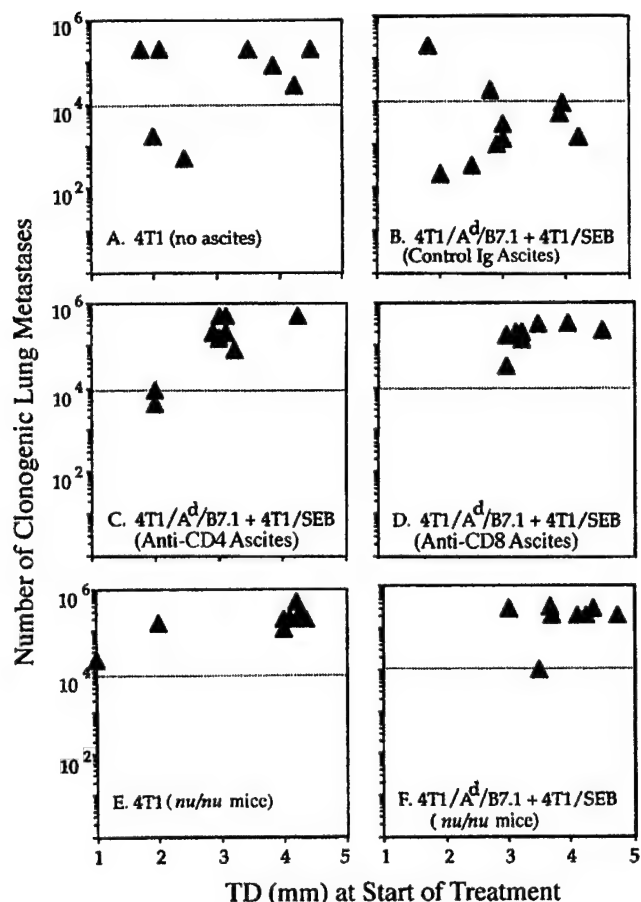


Fig. 5. Reduction of established wild-type metastases with MHC class II, CD80, and SEB immunotherapy requires CD4⁺ and CD8⁺ T cells. A-D, naïve BALB/c mice received injections s.c. in the abdominal mammary gland of 7×10^3 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; A) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (B-D). On days 8, 11, and 13 (i.e., before the start of immunotherapy), mice received injections of either control ascites (10 mice; B), GK1.5 ascites (9 mice; C), or 2.43 ascites (8 mice; D). Antibody injections were continued at least once a week for the duration of the experiment. E and F, BALB/c *nu/nu* mice received injections s.c. in the abdominal mammary gland of 7×10^3 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; E) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (8 mice; F).

CD4⁺ T lymphocytes are involved in immunity induced by MHC class II/CD80 vaccines (15) and that MHC class II/CD80 modified tumor cells function directly as APCs for the initial activation of tumor-specific CD4⁺ T cells (24). Direct presentation of antigen by tumor cells is possible because tumor cell expression of MHC class II molecules in the absence of invariant chain allows for presentation of endogenously synthesized tumor antigens by MHC class II molecules (24, 25). Because the vaccines express MHC class I, class II, CD80, and SEB molecules, antigen-specific and costimulatory signals will be efficiently delivered to CD8⁺ and CD4⁺ T cells. Likewise, because the activated CD8⁺ and CD4⁺ T cells are in close proximity to each other, there should be an efficient transfer of cytokines between CTLs and T helper cells (see Fig. 6, right).

Host-derived APCs are also likely to be involved in CD8⁺ and CD4⁺ T lymphocyte activation during vaccine therapy. Because MHC class II serves as a ligand for a sAg (10), it is likely that any host-derived class II⁺ cell will bind available SEB. The involvement of host APC is supported by the observation that SEB transfectants alone, which do not express MHC class II, cause a modest therapeutic effect (Fig. 4, A versus B). Furthermore, other mouse tumor models have demonstrated that both a class I- and class II-restricted tumor-

encoded antigen can be processed and presented indirectly by host-derived APCs (24, 26, 27). Taken together, it is likely that host-derived APCs, capable of migrating to lymph nodes, coordinately present SEB and tumor antigen to both CD8⁺ and CD4⁺ T cells (see Fig. 6, left).

SEB may also enhance vaccine efficacy because it induces an inflammatory response that stimulates immunity (28). Gene transfer techniques have demonstrated that *in vivo* expression of various sAg (SEA, SEB, and TSST-1) DNAs induces intense inflammatory responses (29). Although systemic administration of sAg (doses >500 μ g) typically triggers T-cell release of cytokines such as tumor necrosis factor and lymphotoxin that lead to cachexia (11), we did not see any adverse side effects in SEB-treated mice.

When a sAg, such as SEB, is coexpressed by the MHC class II/CD80 vaccine, additional activation and/or proliferation signals may be delivered to the specifically activated CD8⁺ and CD4⁺ T cells. Because SEB binds to the sides of MHC class II molecules and the T cell receptor while antigenic peptide binds within the MHC class II cleft (30, 31), it is feasible that the sAg, tumor antigen-specific, and costimulatory signals are simultaneously received by the T cells. Whereas it is also possible that those signals are not coincident, several studies have shown that activation of T cells by SEB is facilitated or enhanced by B7/CD28 signaling (32-35). Controversy exists over the ability of costimulation to inhibit sAg-induced apoptosis, but one report demonstrates that lipopolysaccharide activation of B cells prevents sAg-induced deletion (36). Regardless of the precise kinetics in which the various activation signals are delivered, coordinate delivery of the three signals improves the efficacy of the vaccines to reduce spontaneous metastatic tumor growth. As a result, T-cell activation may be exceptionally efficient because both direct and indirect antigen presentation occur, thus yielding larger numbers of precisely those CD8⁺ and CD4⁺ tumor-specific cells that mediate tumor cell destruction.

New immunotherapies are routinely tested in experimental animal tumor systems. Although such experiments may provide "promising" therapeutic results, tumor regression in animal models does not necessarily predict successful treatment of tumors in human patients. There may be significant physiological and biochemical differences between animals and humans that preclude direct comparison of

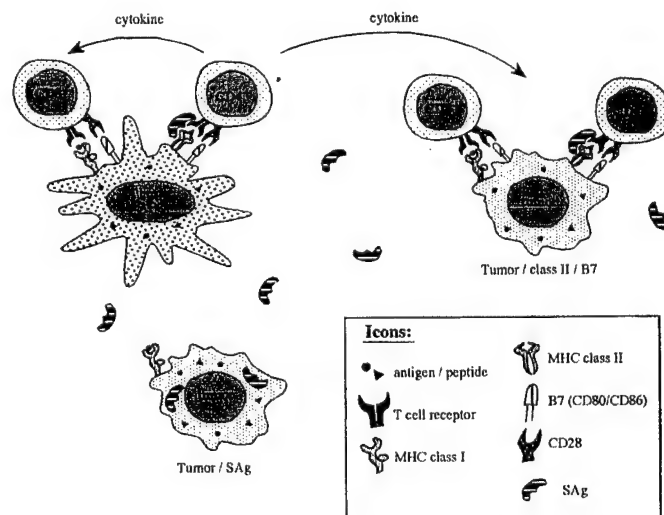


Fig. 6. Proposed mechanism of action by the MHC class II/CD80 and SEB tumor cell-based vaccine. The SEB modified tumor cell (bottom) secretes sAg at the immunization site where the host professional APC (left) and the MHC class II/CD80 modified tumor cell (right) are able to bind the sAg and activate both CD4⁺ and CD8⁺ T cells. As a result, immunization occurs by direct and indirect (cross-priming) antigen presentation.

results, and testing in humans, therefore, will always be required. However, the inability to translate therapies developed in experimental animal models to humans may also be because many of the mouse tumor systems used in immunotherapy studies do not closely model human cancers and, hence, the immunotherapies are not being tested in clinically relevant settings. For example, many immunotherapies are tested in so-called "metastatic" settings, however: (a) the extent of metastatic disease is minimal; (b) the metastases have not arisen spontaneously; and/or (c) the metastases have not been established for significant amounts of time. Furthermore, many commonly used mouse models: (a) are not spontaneously metastatic (e.g., CMS-5 fibrosarcoma, RENCA renal cell carcinoma, CT-26 colon adenocarcinoma, Sa1 sarcoma, and so forth); (b) rapidly lose their metastatic potential when cultured *in vitro* (e.g., K1735 melanoma); (c) metastasize poorly unless the primary tumor is excised (e.g., B16 melanoma, line 1 carcinoma); or (d) rapidly invade the local environment, such that animals die from primary tumor before metastatic disease is established (e.g., B16 melanoma). In contrast, the 4T1 mammary carcinoma is spontaneously metastatic and metastasizes to many of the organs to which human breast cancer metastasizes (e.g., lung, liver, and brain). Also, similar to human mammary carcinoma, 4T1 metastases spread and progress while primary tumor is in place. In addition, following inoculation of a small number of tumor cells (7×10^3) in the mammary gland, lethal metastatic disease develops early (within the first 2–3 weeks) and progresses over several weeks so that immunotherapies can be tested against early or very advanced stage disease. The 4T1 tumor, therefore, is an excellent model for testing experimental immunotherapies. In contrast to our earlier studies with the 4T1 tumor in which relatively early metastases were treated and primary tumor was left in place (3), the studies reported here address very advanced metastatic disease in a postsurgical setting. Although the statistically significant extension of survival time following surgery and administration of immunotherapy was small, we find no comparable studies in the literature in which the efficacy of an immunotherapy is demonstrated in such a clinically relevant model of advanced stage metastatic disease.

ACKNOWLEDGMENTS

We thank Drs. T. Iamonte-Armstrong and B. Bradley for assistance with statistical analysis and S. Mason for animal care.

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ORIGINAL ARTICLE

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Immunotherapy with vaccines combining MHC class II/CD80⁺ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon γ

Received: 15 October 1999 / Accepted: 24 November 1999

Abstract Because they are difficult to treat, animal models of widespread, established metastatic cancer are rarely used to test novel immunotherapies. Two such mouse models are used in this report to demonstrate the therapeutic efficacy and to probe the mechanisms of a novel combination immunotherapy consisting of the cytokine interleukin-12 (IL-12) combined with a previously described vaccine based on MHC class II, CD80-expressing cells. BALB/c mice with 3-week established primary 4T1 mammary carcinomas up to 6 mm in diameter and with extensive, spontaneous lung metastases show a significant reduction in lung metastases following a 3-week course of immunotherapy consisting of weekly injections of the cell-based vaccine plus injections of IL-12 three times per week. C57BL/6 mice with 7-day established intravenous B16 melF10 lung metastases show a similar response following immunotherapy with IL-12 plus a vaccine based on B16 MHC class II, CD80-expressing cells. In both systems the combination therapy of cells plus IL-12 is more effective than IL-12 or the cellular vaccine alone, although, in the 4T1 system, optimal activity does not require MHC class II and CD80 expression in the vaccine cells. The cell-based vaccines were originally designed to activate tumor-specific CD4⁺ T lymphocytes specifically and thereby provide helper activity to tumor-cytotoxic CD8⁺ T cells, and IL-12 was added to the therapy to facilitate T helper type 1 lymphocyte (Th1) differentiation. In vivo depletion experiments for CD4⁺ and CD8⁺ T cells and natural killer (NK) cells and tumor challenge

experiments in beige/nude/XID immunodeficient mice demonstrate that the therapeutic effect is not exclusively dependent on a single cell population, suggesting that T and NK cells are acting together to optimize the response. IL-12 may also be enhancing the immunotherapy via induction of the chemokine Mig (monokine induced by interferon γ), because reverse PCR experiments demonstrate that Mig is present in the lungs of mice receiving therapy and is most likely synthesized by the tumor cells. These results demonstrate that the combination therapy of systemic IL-12 and a cell-based vaccine is an effective agent for the treatment of advanced, disseminated metastatic cancers in experimental mouse models and that multiple effector cell populations and anti-angiostatic factors are likely to mediate the effect.

Key words Immunotherapy · Metastatic mammary carcinoma · IL-12 · Angiogenesis · CD80 · MHC class II · CD4⁺ T cells

Introduction

Many of the recently explored immunotherapy strategies for the treatment of cancer have focused on the improved activation of tumor-specific immunity. For example, administration of interleukin-12 (IL-12), a cytokine that favors T helper type 1 lymphocytes (Th1) and natural killer (NK) cell development and stimulates anti-angiogenic chemokines [12, 20], reduces tumor burden in numerous mouse tumor systems [4, 38, 39]. Likewise, the treatment of mice with established primary and/or metastatic tumor with irradiated immunogenic tumor cells, constitutively expressing MHC class I molecules and transfected/transduced with the costimulatory molecule CD80, reduces primary tumor mass and/or small metastatic tumor load [6, 41]. This latter approach is based on the premise that the genetically engineered tumor cells present both antigen-specific and costimulatory T cell activation signals to the relevant CD8⁺ T lymphocytes.

This work was supported in part by US Army Research and Materiel Command DAMD17-94-J-4323 and NIH R01CA52527. B. Pulaski is support by a post-doctoral fellowship from the US Army Research and Development Command, DAMD17-97-1-7152

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Another immunotherapeutic strategy aimed at specifically improving the generation of tumor-specific CD4⁺ T lymphocytes uses autologous tumor cells transfected with syngeneic MHC class II genes plus CD80 costimulatory molecule genes as cell-based vaccines for the treatment of mice with established primary and metastatic cancer. This therapy is based on the hypothesis that enhanced generation of CD4⁺ tumor-specific T helper lymphocytes facilitates CD8⁺ T cell activation and promotes stable, long-term immune memory against recurrence of primary tumor and/or outgrowth of micrometastases [27, 28]. Treatment with MHC-class-II-transfected-cell-based vaccines has yielded significant reductions in solid tumor mass [1, 2] and in established, spontaneous metastatic disease [32].

In an attempt to generate a more potent antitumor effect, IL-12 and CD80 therapies have been combined to target the activation of CD8⁺ T cells. Because in vitro studies have shown that IL-12 plus CD80 produces optimal T cell proliferation and interferon γ (IFN γ) production [23, 25] as well as stimulating a primary anti-tumor response in vitro [17], it is not surprising that IL-12 and CD80 synergize to bring about significant regression of established primary tumor as well as inducing immunological memory against recurrence of primary tumor [11, 33]. Although a principal function of IL-12 is its ability to promote CD4⁺ Th1 differentiation, surprisingly, IL-12 therapy has not previously been combined with other therapies that specifically target the activation of tumor-specific CD4⁺ T cells. To test the potential effect of targeting with IL-12 plus CD4⁺, we have combined systemic IL-12 therapy with immunization using MHC class II/CD80 genetically modified tumor cells for the treatment of established (induced i.v. and spontaneous) metastatic disease. Previous in vivo studies testing IL-12 therapy have used mouse tumor models consisting of either solid, subcutaneous primary tumors, or very early metastases induced by intravenous injection of malignant cells [4, 7, 9, 10, 11, 31, 33]. Although these model systems provide some insight into the potential role of therapeutic agents in the treatment of cancer, they are not realistic clinical situations in which larger metastatic tumor loads are likely to be encountered and for which more effective treatments are needed.

To test potential immunotherapies more rigorously against larger metastatic loads, we have used two mouse tumor systems. The 4T1 mammary carcinoma tumor is a very poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes to lymph nodes, lung, liver, brain, and blood following growth of the primary tumor in the mammary gland [24, 32]. This disease progression closely parallels human breast cancer and makes the 4T1 tumor an excellent model for human disease [32] and a rigorous animal model of advanced spontaneous metastatic disease.

As a second model system, we have used the B16-derived melF10 melanoma [16]. This tumor is also very poorly immunogenic and highly malignant, and metastasizes immediately to the lung when inoculated intra-

venously. Similar to approximately 15% of human cancers, melF10 has markedly reduced levels of MHC class I molecules. This phenotype probably contributes to its reduced immunogenicity and heightened tumorigenicity. Many investigators have used the melF10 tumor as an experimental model; however, most studies use lung metastases very early after they are established (e.g. 3 days or less after i.v. inoculation). We have used longer-established melF10 lung metastases (therapy begins on day 7 after i.v. inoculation) to test the combination vaccine more rigorously. For both tumors, the combined therapy is more effective than either therapy alone, and appears to be mediated by multiple independent effector mechanisms including T lymphocytes, NK cells, and possibly chemokine production that has been linked to anti-angiogenesis.

Materials and methods

Cells and transfectants

MelF10 is a high metastatic variant of the C57BL/6-derived B16 melanoma [16]. 4T1 is a spontaneously metastatic, poorly immunogenic BALB/c-derived mammary carcinoma [24]. Culture conditions for both tumors have been previously described [29, 32]. Generation and characterization of 4T1 transfectants expressing I-A^d and CD80 and B16melF10 transfectants expressing I-A^b and CD80 have been previously described [29, 32].

Mice Mice were purchased from The Jackson Laboratory (Bar Harbor, Me.) or bred in the UMBC Animal Facility from breeding pairs purchased from The Jackson Laboratory. Experiments using the 4T1 mammary carcinoma or melF10 melanoma were performed in female BALB/c mice and C57BL/6 male or female mice, respectively. All mice were between 6 weeks and 6 months in age.

Tumor challenges and metastases assays

Tumorigenesis and metastasis formation by the melF10 [29] and 4T1 [32] tumors were performed as previously described. Briefly, for experimental metastases, 10⁵ melF10 cells/100 μ l for each mouse were inoculated intravenously (i.v.) into the tail vein of C57BL/6 mice on day 0; the mice were sacrificed 3–4 weeks later and their lungs observed and weighed. For spontaneous metastases 7 \times 10³ 4T1 cells/50 μ l for each mouse were inoculated into the abdominal mammary gland of BALB/c female mice on day 0; the mice were sacrificed 6 weeks later and the number of clonogenic metastatic cells in the lungs assessed by growth in medium supplemented with 6-thioguanine [32]. Mice carrying tumors were closely followed for symptoms of pain and distress and were sacrificed when they became moribund. On the basis of previous studies [32], 4T1-bearing mice with up to 10 000 clonogenic metastatic 4T1 cells in their lungs are considered responder mice. All animal procedures followed the *Principles of laboratory animal care* (NIH publication 85-23, revised 1985) and were approved by the UMBC Institutional Animal Care and Use Committee.

Antibodies

Protein A or protein G purification of MHC-class-II-specific mAb 3JP (I-A^b [19]), MKD6 (I-A^d [21]), MHC-class-I-specific mAb 20-8-4 (H-2 K^b [30]), 28-14-8 (H-2D^b [30]), CD4 (GK1.5 [42]), and CD8 (2.43 [35]) was as previously described [29, 32]. Fluorescently coupled CD3, CD4, CD8, NK1.1, and B220 mAb were purchased from Pharmingen.

In vivo depletions for CD4⁺ and CD8⁺ T cells and NK cells

Depletions of CD4⁺ and CD8⁺ T cells were performed as previously described [2]. Briefly, mAb to CD4⁺ (GK1.5) and CD8⁺ (2.43) T lymphocytes were prepared as ascites fluid in nude mice and had titers of at least 1/80 000 as measured by immunofluorescence. Mice were inoculated intraperitoneally (i.p.) with 100 μ l (GK1.5) or 150 μ l (2.43) ascites fluid mixed with an equal volume of phosphate-buffered saline on days -6, -3, and -1 before tumor challenge, and then once a week during tumor growth. Depletion resulted in elimination of 90%–100% of splenic CD4⁺ T cells and 100% of splenic CD8⁺ T cells as measured by immunofluorescence at the conclusion of the experiment.

BALB/c mice were depleted for NK cells by i.p. inoculation with 40 μ l anti-asialoGM1 antiserum (Wako Pharmaceuticals; reconstituted as directed by manufacturer) on days -4 and -1 before therapy began and twice a week while therapy continued until the day of sacrifice. To ascertain NK depletions, anti-asialoGM1-treated mice and control rabbit-Ig-treated mice were boosted with 1 mg poly(I.C) i.p. at the conclusion of the experiment and, 24 h later, their spleens were tested for NK activity by ⁵¹Cr-release assay. At E:T ratios of 100:1, 50:1, and 25:1, anti-asialoGM1-depleted mice yielded 12%, 1%, and 0% cytotoxicity, respectively, compared to control rabbit-antibody-treated mice, which yielded 22%, 8%, and 6% cytotoxicity, respectively, against YAC-1 targets.

NK assays

NK assays were performed as previously described [26] using P815 cells as NK-resistant targets and YAC-1 cells as NK-susceptible targets. Briefly, target cells (up to 10⁷) were radiolabeled with ⁵¹Cr for 1.5 h and chased for 30 min at 37 °C in a volume of 0.5 ml. Labeled targets (5 \times 10⁴/well) were incubated with splenocytes (effectors) at ratios ranging from 100:1 to 6.25:1 in a final volume of 200 μ l/well in 96-well plates. Cells were incubated at 37 °C for approximately 4 h, and supernatants harvested and counted in a Wallac minigamma counter. The percentage chromium release was calculated as [experimental release (cpm) – spontaneous release (cpm)]/[total release (cpm) – spontaneous release (cpm)] \times 100. Mice were induced for NK activity by i.p. inoculation of 100 μ l poly(I.C) 24 h prior to removal of spleens.

IL-12 and cell therapy

IL-12 therapy consisted of i.p. inoculations of 1 μ g/mouse three times per week for 3 weeks. This dose was based on previous studies [39]. Cell therapy consisted of i.p. inoculations of 10⁶ irradiated (50 Gy) melf10, 4T1, melf10 transfectants, or 4T1 transfectants once a week for 3 weeks. Therapy for 4T1 and melf10 experiments was started 21 and 7 days after wild-type tumor challenge respectively.

In vitro and in vivo detection of monokine induced by IFN γ (Mig) by reverse polymerase chain reaction (PCR)

4T1 and Melf10 cell lines were induced in vitro with 100 U/ml interferon γ (IFN- γ) for 2 h. Naive mice were injected with 4T1 parental tumor and treated with immunotherapy as described above. Lung tissue was removed at the indicated times after the start of immunotherapy treatment. RNA was isolated from all samples by using RNA-STAT and DNase-treated with RQ1 RNase-free DNase as directed by the manufacturer. cDNA was generated using dT₁₂₋₁₈ primers and murine Moloney leukemia virus reverse transcriptase. Semi-quantitative PCR amplification for β -actin and Mig was performed using the following primer pairs:

MuMig primers : forward (5'GATCAAACCTGCCTAGA3');
reverse (5'CTTGAACGACGACGAC3')

β -actin primers : forward(5'GTCCCTGTATGCCTCTG3');
reverse(5'ATGAGGTAGTCTGTCTCAGGT3')

Statistical analyses

To determine the statistical significance of the data, Tukey's Honestly Significant Difference test was performed at a *P* value set at 0.05. Tukey's test is a multi-comparison test that determines the statistical significance of three or more data sets and allows for unequal sample size (*n*) and sample variances [44].

Results

Treatment of mice with established B16-melf10 lung metastases with transfected tumor cells plus IL-12 reduces metastatic disease

To test the combination therapy of IL-12, MHC class II, and CD80, we transfected the melf10 tumor with syngeneic MHC class II genes (Aa^b and Ab^b genes encoding the I-A^b class II molecule) plus the CD80 gene. Transfectants were screened by indirect immunofluorescence for class II and/or CD80 expression and cloned by limiting dilution.

Figure 1 shows the flow-cytometry profiles of melf10 cells transfected with MHC class II genes (melf10/A^b), the CD80 gene (melf10/B7), or both class II plus CD80 (melf10/A^b/B7) and stained with mAb for MHC class I, class II, or CD80. Wild-type melf10 and the transfectant cells have relatively low levels of endogenously encoded MHC class I molecules (H-2K^b and H-2D^b; 20–8–4 and 28–24–8 mAb; Fig. 1i–l and m–p respectively). I-A^b and CD80 are only expressed on those cell lines carrying these transgenes (Fig. 1b, d and g, h respectively).

Because our goal is to generate more effective therapeutic strategies, the therapeutic efficacy of the melf10 transfectants plus IL-12 was tested in mice with advanced established lung metastases. Syngeneic C57BL/6 mice were inoculated i.v. in the tail vein with 10⁵ wild-type melf10 cells. Following i.v. injection the melf10 cells rapidly migrate to the lungs, and mice die from metastatic lung tumor within 3–4 weeks (V. Clements and S. Ostrand-Rosenberg, unpublished results). On day 7 after inoculation of wild-type tumor, immunotherapy was started. Each mouse was given one injection/week of 10⁶ irradiated tumor cells or transfectants and three injections/week of 1 μ g IL-12. Therapy was continued for 3 weeks; the mice were then sacrificed and the lungs removed, visually inspected, and weighed. Figure 2 shows the lungs of treated mice from one typical experiment and Table 1 shows the pooled results of three experiments in which tumor-bearing mice were treated with IL-12 \pm melf10, melf10/A^b, melf10/B7, or melf10/A^b/B7 cells. By visual inspection of the lungs (Fig. 2) and by comparing the average of mean lung weights (Table 1), the greatest reduction in lung metastases can be seen in mice treated with melf10/A^b/B7 cells plus IL-12. Therapy with IL-12 alone also reduces lung metastases, as does therapy with melf10/A^b cells

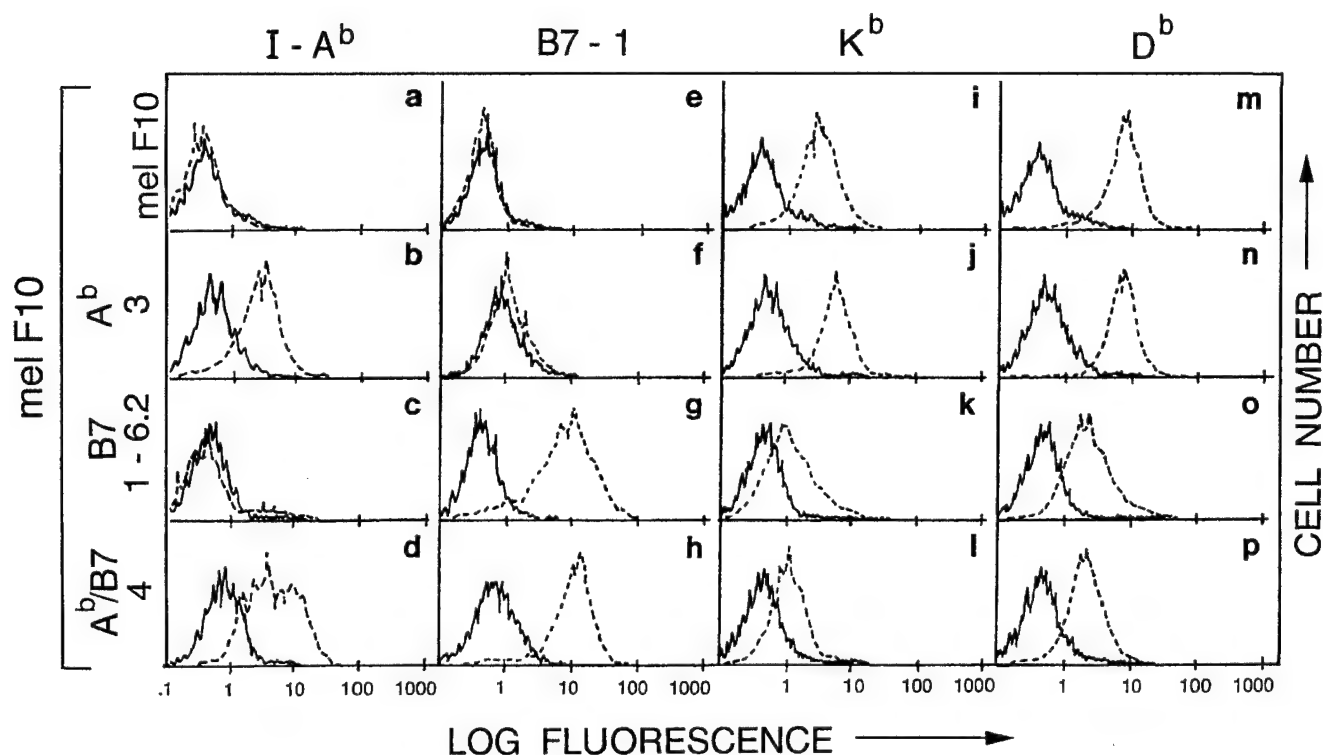


Fig. 1 Flow-cytometry profiles of melF10 and melF10 transfectants stained for MHC class I (H-2 K^b and H-2D^b), MHC class II (I-A^b), and B7.1 (CD80) molecules. *Dotted lines* staining by specific antibody plus fluorescent conjugate; *solid lines* staining by fluorescent conjugate alone

plus IL-12 or therapy with melF10/B7 cells plus IL-12. Therapy with transfectants alone does not have a measurable effect (Table 1). Statistical analysis of lung weights (Table 1), using Tukey's test at $P = 0.05$,

demonstrates a significant difference between the treatment groups (IL-12 alone or IL-12 plus any cell combination) and the untreated group or the group treated with cells alone; however, the analysis does not demonstrate a statistically significant difference between the group receiving IL-12 alone and those treated with IL-12 plus any of the cell-based vaccines. The absence of statistical significance between these groups is most likely due to the approximation of metastatic cell content by

Fig. 2 Metastatic tumor cells in the lungs of C57BL/6 mice treated with cell and/or interleukin-12 (IL-12) therapies. Mice were inoculated i.v. on day 0 with wild-type melF10 tumor cells and therapy started on day 7. The therapy for each group is indicated. Following 3 weeks of therapy, lungs were excised. Each set of lungs is from an individual mouse

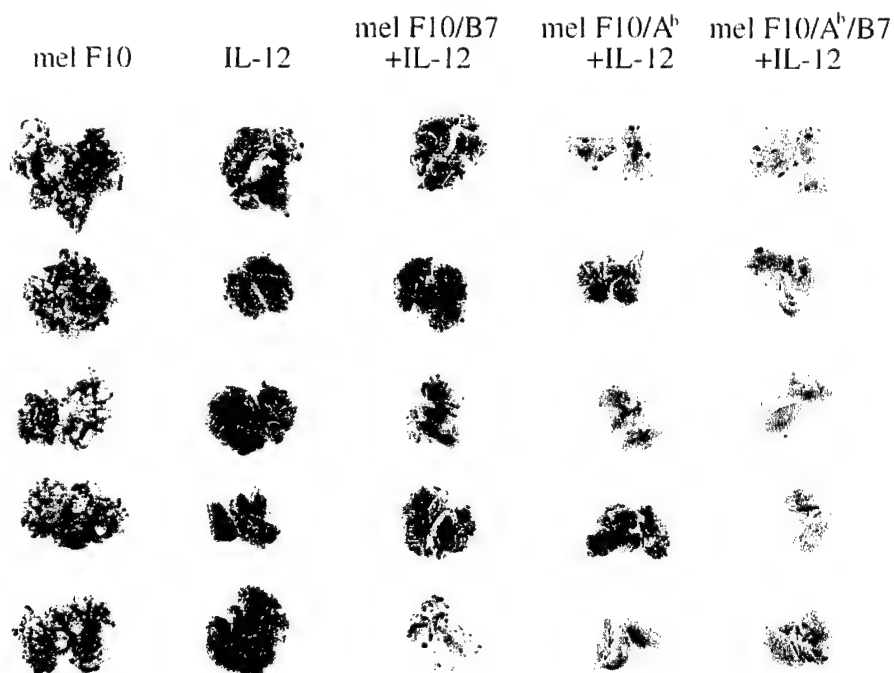


Table 1 Mice carrying established i.v. B16melF10 melanoma metastases have reduced lung metastases following treatment with melF10/A^b/B7.1 plus interleukin-12 (IL-12). Syngeneic C57BL/6 J mice were challenged i.v. on day 1 with 10⁵ wild-type B16melF10 tumor cells. Beginning 7 days after tumor inoculation, and con-

tinuing for the following 3 weeks, mice were inoculated i.p. once a week with therapeutic cells and three times per week i.p. with 1 µg IL-12. Surviving mice were sacrificed on day 26 after initial tumor inoculation and lung weights determined

Treatment				Mean lung weight (mg) (± SD)
Therapeutic cells	Number mice per group	IL-12	T cell depletion	
–	9	–	–	1011 ± 213
melF10/A ^b /B7.1	5	–	–	990 ± 169
–	10	IL-12	–	464 ± 124*
melF10	5	IL-12	–	313 ± 31*
melF10/A ^b	5	IL-12	–	337 ± 55*
melF10/B7.1	5	IL-12	–	409 ± 35*
melF10/A ^b /B7.1	15	IL-12	–	352 ± 205*
melF10/A ^b /B7.1	5	IL-12	CD4 depleted	250 ± 41*
melF10/A ^b /B7.1	5	IL-12	CD8 depleted	325 ± 13*

* These groups are statistically significantly different from the untreated groups and the groups treated with melF10/A^b/B7.1 cell vaccine alone (Tukey's test, $P = 0.05$)

lung weight, which can only detect gross differences. Therefore, by visual inspection, the therapy that shows the greatest reduction in lung metastases is treatment with IL-12 plus the MHC class II/CD80 vaccine.

Mice carrying established 4T1 mammary carcinoma metastases have reductions in metastatic disease following treatment with IL-12 plus 4T1 transfectants or wild-type tumor

To determine if the effect of therapy on MelF10 tumors is applicable to additional tumors, we performed similar experiments with the 4T1 tumor in which the number of metastatic cells can be very precisely quantified [32]. Syngeneic BALB/c female mice were inoculated in the mammary gland with 7×10^3 4T1 tumor cells and tumors allowed to develop for 3 weeks, at which time the primary tumors ranged from 1 mm to 8 mm in diameter. Previous experiments ascertained that, at this time and size of primary tumor, extensive metastatic disease is well established in the lungs, and that the number of metastatic cells in the lungs is proportional to the size of the primary tumor [32].

Therapy was started on day 21 after the initial tumor challenge and consisted of one injection per week of 10⁶ irradiated tumor cells or transfectants (4T1, 4T1/A^d, 4T1/B7.1, or 4T1/A^d plus 4T1/B7.1) plus three injections/week of 1 µg IL-12/mouse. Mice were sacrificed after 3 weeks of therapy (day 42 after initial 4T1 inoculation) and lung tissue was dissociated into single-cell suspensions and plated in medium containing 6-thioguanine. Ten days later the number of clonogenic metastatic 4T1 cells was counted because 4T1 cells are resistant to 6-thioguanine and normal cells are killed by the drug [32].

Figure 3 shows the results of the 4T1 therapy experiments. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results are plotted as the number of metastatic cells in

the lung at the end of treatment versus the size of primary tumor when the therapy is started. A line denoting a level of 10 000 tumor cells in the lungs is also included because almost all untreated mice contain more than 10 000 metastatic cells in their lungs after 42 days of primary tumor growth [32]. As shown in Fig. 3, therapy with unmodified tumor (Fig. 3A) or 4T1/A^d plus 4T1/B7.1 transfectants (Fig. 3B) minimally reduces the number of mice with more than 10 000 metastases in the lungs (69% and 67% respectively). Treatment with IL-12 alone (Fig. 3C) causes some reduction in the number of metastatic cells relative to control 4T1-treated mice (Fig. 3A); however, 41% of the IL-12-treated mice still have more than 10 000 metastatic cells in their lungs. In contrast, treatment with IL-12 plus any transfectant or IL-12 plus wild-type 4T1 results in only 11%–25% of mice having more than 10 000 metastatic cells in the lungs (Fig. 3D–G). To analyze the statistical significance of these results, we used the Tukey's test at $P = 0.05$. Because the individual treatment groups involving cells plus IL-12 (Fig. 3D–G) do not statistically differ from each other, the results of these groups were pooled. According to Tukey's test, the pooled results from the groups treated with cells plus IL-12 (Fig. 3D–G) are significantly different from those of groups receiving cell therapy alone (Fig. 3A, B), and IL-12 therapy alone does not give significantly different results from those of any treatment group. Maximum reduction of established, spontaneous 4T1 lung metastases, therefore, occurs following combination therapy of IL-12 plus irradiated tumor cells; however, wild-type tumor cells are as effective as transfectants in the combined therapy.

Combination IL-12 and cellular therapy affects the growth of smaller primary, solid tumors

Previous studies have demonstrated that IL-12 therapy mediates tumor regression of relatively small, primary,

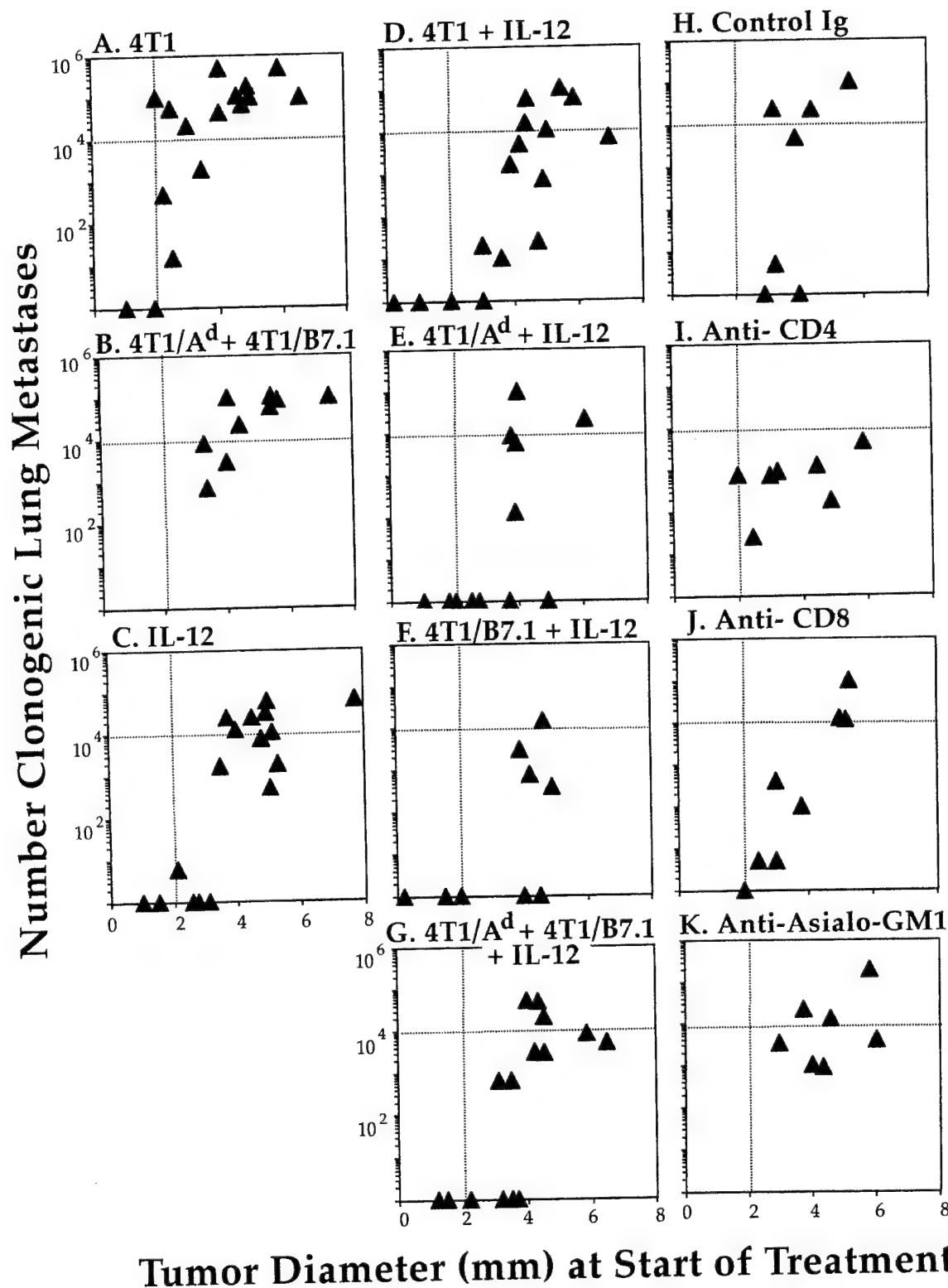


Fig. 3 Number of metastatic cells in BALB/c mice carrying established wild-type 4T1 metastases and treated with cell and/or IL-12 therapies. The therapy for each group is indicated within each panel. Mice were inoculated with wild-type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse. To determine statistical significance, the number of clonogenic lung metastases were transformed to log₁₀ values and analyzed using Tukey's test ($P = 0.05$)

solid tumors. To confirm these observations and to determine whether the presence of metastatic disease complicates the effect of IL-12 therapy on primary solid tumor, we have also analyzed primary tumor growth in the animals used for the results in Fig. 3. Primary tumor diameters were measured at the beginning of therapy and two or three times per week thereafter. At the end of

3 weeks of therapy, mice were categorized as follows: responding mice showed a marked reduction in primary tumor diameter, non-responding mice showed continued growth of primary tumor, partially responding mice showed no change in tumor size. To determine whether primary tumor burden at the start of therapy affects therapeutic outcome, mice were analyzed according to tumor diameter at the start of treatment. As shown in Table 2, mice treated with cells only (4T1 or 4T1/A^d + 4T1/B7.1) are uniformly non-responders. All mice with small tumors at the start of treatment (0–1.99 mm in diameter), regardless of the therapy (IL-12 alone or IL-12 plus cells), are responders. In contrast, mice with larger tumors (above 2 mm in diameter) at the start of therapy responded heterogeneously. For mice with tumors of 2–3.9 mm, IL-12 plus 4T1/A^d/B7.1 cells produced the largest number of responding mice; however all therapies, including IL-12 (IL-12 alone or IL-12 plus any cells), produced approximately equal responses if the results of responding and partially responding mice are pooled. In contrast, there were fewer responding and partially responding mice if therapy was started when tumors were 4–5.9 mm in diameter, and responses to IL-12 alone or IL-12 plus cells did not differ. Among mice starting with tumors more than 6 mm in diameter there were no responders or partial responders. In agreement with studies by other investigators, IL-12 therapy alone, therefore, mediates regression or partial regression of tumors less than 6 mm in diameter in a subset of treated mice. However, combination therapy of IL-12 plus tumor cells causes more complete responses for tumors that are 2–4 mm in diameter at the start of therapy.

Immunotherapy effect is not exclusively dependent on CD4⁺, CD8⁺, or NK cells

The concept of combining a cell-based immunization therapy with IL-12 was based on the hypothesis that

IL-12 facilitates the development of Th1-type CD4⁺ T helper lymphocytes [18] that are activated by immunization with the MHC class II⁺B7.1⁺ tumor cells. These Th1 cells would, in turn, provide "help" to tumor-specific CD8⁺ T lymphocytes and improved antitumor immunity would result. To test this hypothesis and to determine the involvement of T cells, mice undergoing immunotherapy were depleted of CD4⁺ and CD8⁺ T cells. Depletions were started prior to the first cycle of immunotherapy. These experiments were completed in both the melf10 and 4T1 systems, and the immunotherapy protocols for each tumor were identical to those used in the experiments of Table 1 (B16melf10 tumor) and Fig. 3 (4T1 tumor). As shown in Table 1, and Fig. 3H–J, depletion of CD4⁺ or CD8⁺ T cells does not affect metastatic growth in mice treated with MHC class II⁺B7.1⁺ cells plus IL-12, indicating that neither of these populations by itself is responsible for the therapeutic effect.

In addition to its role in Th1 development, IL-12 also stimulates NK cell function [3]. Mice with the melf10 tumor and undergoing immunotherapy, therefore, were also tested for splenic NK cell levels and NK activity. Splenocytes of C57BL/6 mice carrying established melf10 metastases, and having received at least 2 weeks of immunotherapy were tested for NK1.1, CD4⁺, CD8⁺, CD3⁺, and B220⁺ expression. The percentages of CD4⁺, CD8⁺, and B220⁺ splenocytes did not change following therapy with cells and/or IL-12 (data not shown). In contrast, as shown in Table 3, the percentage of NK1.1⁺ splenocytes was statistically significantly higher in all treated mice than in naive untreated and tumor-bearing control mice (Tukey's $P = 0.05$). Therapy with any of the cell-based vaccines and/or IL-12, therefore, significantly increases the number of NK cells in the spleen.

Recent studies have attributed IL-12-mediated tumor rejection to a novel population of NK⁺CD3⁺ MHC-unrestricted effector cells [13]. Splenocytes from melf10-

Table 2 IL-12 alone and IL-12 plus cells mediate regression of primary tumors of 4 mm or less
ND not determined; *R* responder, primary tumor diameter regressed during therapy; *PR* partial responder, primary tumor diameter remained the same during therapy; *NR* non-responder, primary tumor diameter continued to grow during therapy

Therapeutic cells	IL-12	No. responding mice/total mice treated, for mice having a tumor diameter at start of therapy (mm) of:			
		0–1.99	2–3.99	4–5.99	> 6
4T1	–	2/2 NR	6/6 NR	7/7 NR	1/1 NR
4T1/A ^d + 4T1/B7.1	–	ND	2/2 NR	6/6 NR	1/1 NR
–	IL-12	2/2 R	1/7 R 4/7 PR 2/7 NR	2/8 PR 6/8 NR	2/2 NR
4T1	IL-12	2/2 R	2/6 R 3/6 PR 1/6 NR	8/8 NR	1/1 NR
4T1/A ^d	IL-12	1/1 (R)	3/8 R 4/8 PR 1/8 NR	2/2 NR	1/1 NR
4T1/B7.1	IL-12	2/2 (R)	1/3 R 1/3 PR 1/3 NR	2/5 R 3/5 NR	ND
4T1/A ^d + 4T1/B7.1	IL-12	2/2 (R)	4/6 R 1/6 PR 1/6 NR	2/6 PR 4/6 NR	1/1 NR

bearing mice, therefore, were doubly stained for NK1.1 and CD3. As shown in Table 3, naive (non-tumor-bearing) and untreated tumor-bearing mice have lower levels of NK1.1⁺CD3⁺ cells than do mice receiving IL-12 and/or vaccine therapy. Statistical analysis using Tukey's test at $P = 0.05$ demonstrates statistically significant differences between the naive and untreated tumor-bearing groups, the groups treated with melF10 \pm IL-12, and the group receiving melF10/A^b/B7 plus IL-12. Therapy of tumor-bearing mice with IL-12 and/or tumor cells, therefore increases NK⁺CD3⁺ cells, and the greatest increase is for mice treated with IL-12 plus the vaccine of class-II⁺CD80⁺ cells, suggesting that NK⁺CD3⁺ cells may be involved in the therapeutic effect.

To determine if the increase in splenic NK levels resulted in an increase in functional NK cell activity, splenocytes from mice treated with melF10 and naive mice were tested in vitro as effector cells in NK assays using P815 cells as NK-resistant and YAC-1 cells as NK-sensitive targets. Tumor-free, untreated C57BL/6 mice were given poly(I,C) 24 h before assay to stimulate NK activity and served as positive controls for measuring NK functional activity. As shown in Table 3, treated mice did not have higher NK functional activity than untreated mice, while poly(I,C)-boosted mice showed NK activity: 30% killing at E:T ratios of 50:1 splenocytes:YAC-1 targets. Cytotoxicity against P815 cells was less than 5%. Mice treated with IL-12 and/or cell-based immunotherapies, therefore, have increased phenotypic levels of NK⁺ splenocytes, but these splenocytes do not display increased cytotoxic function as measured in vitro.

To determine if NK cells are responsible for the anti-metastatic effects of the cell-based vaccine plus IL-12 in the 4T1 system (Fig. 3D–G), BALB/c mice carrying 4T1 primary tumors were depleted of NK cells by anti-asialoGM1 polyclonal antibodies prior to initiation of 4T1/A^d plus 4T1/B7 plus IL-12 therapy. As shown in Fig. 3, treated mice depleted of NK cells (Fig. 3K) have more metastatic tumor cells in their lungs than non-NK-depleted mice (Fig. 3G, H); however, they do not

have levels of metastatic cells as high as 4T1-treated mice (Fig. 3A). Statistical analysis of these data by Tukey's test does not demonstrate a significant difference between the non-depleted treated mice (Fig. 3G, H) and the asialoGM-1-depleted treated mice (Fig. 3K). NK cells, therefore, may mediate some of the therapeutic effect; however, other effector mechanisms are probably also involved.

Although neither T nor NK cells are exclusively responsible for the therapeutic effect, these cell populations may be functioning cooperatively to diminish growth of metastatic cells. To test this hypothesis, beige/nude/XID mutant mice, which are deficient in NK and T cells, were challenged with 4T1 tumor in the mammary gland and given therapy with 4T1 cells alone, IL-12 alone, or 4T1 cells plus IL-12 initiated on day 21. As shown in Fig. 4, therapy with IL-12 alone (Fig. 4B) or IL-12 plus 4T1 cells (Fig. 4C) resulted in 50% and 33% of mice having more than 10 000 metastatic cells in the lungs, respectively, whereas 100% of mice treated with 4T1 alone (Fig. 4A) had more than 10 000 metastatic cells in the lungs. When compared to immunocompetent mice treated with the same therapies (Fig. 3A, C, D respectively), however, the beige/nude/XID mice have a smaller reduction in the number of clonogenic lung metastases. Beige/nude/XID mice, therefore, respond to the therapy; however, the response is not as great as for immunocompetent mice, suggesting that NK and T cells are partially responsible for the therapy effect.

4T1 and MelF10 tumor cells are induced by IFN γ to express the chemokine Mig

Recent studies indicate that IL-12 and its downstream mediator IFN γ may regulate tumor growth by stimulating anti-angiogenic chemokines including monokine induced by IFN γ (Mig) and IFN γ -inducible protein 10 (IP-10) [12, 20, 40]. Because T and NK cells do not appear to be exclusively responsible for the anti-metastatic response, we hypothesized that chemokines

Table 3 Mice carrying established melF10 metastases and treated with IL-12 and/or cell vaccines have increased levels of splenic natural killer (NK) cells. The two group sizes are for NK1.1 and NK1.1 + CD3⁺ groups respectively. Splenocytes from tumor-free,

untreated mice given poly(I,C) 24 h prior to assay had NK cytotoxicity levels of 30% at 50:1 effector:YAC-1 ratios. The percentage cytotoxicity against P815 cells for all effectors was below 5%

Primary tumor	Number of mice in group	Therapy		NK cells in spleen (%)		Splenic NK activity (%) against YAC-1 at 50:1 E:T ratio
		Cells	IL-12	NK1.1 ⁺	NK1.1 ⁺ + CD3 ⁺	
None	4, 4	–	–	3.8 \pm 0.5	1.5 \pm 0.2	ND
MelF10	4, 2	–	–	3.6 \pm 1.5	2 \pm 0.1	5
	4, 2	–	IL-12	14.9 \pm 4.7*	7.4 \pm 0.4**	2
	4, 4	MelF10	–	10.1 \pm 0.4*	6.3 \pm 0.7**	8
	4, 2	MelF10	IL-12	15.6 \pm 9*	8 \pm 2**	ND
	5, 2	MelF10/A ^b /B7.1	IL-12	15.1 \pm 8.8*	13 \pm 0.3***	2

* Statistically significantly different from naive and tumor-bearing untreated control groups for NK1.1 analysis (Tukey's test $P = 0.05$)

** Statistically significantly different from naive and tumor-bearing untreated control groups and the group receiving melF10/A^b/B7.1 plus IL-12 therapy for NK1.1⁺ + CD3⁺ analysis (Tukey's test $P = 0.05$)

*** Statistically significantly different from all groups for NK1.1⁺ + CD3⁺ analysis (Tukey's test $P = 0.05$)

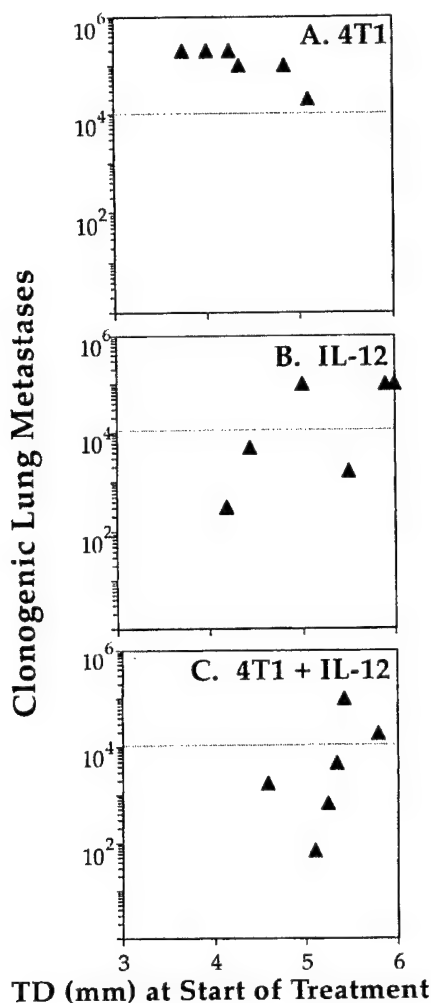


Fig. 4 Number of metastatic cells in CR:NIH-beige/nude/XID mice carrying established wild-type 4T1 metastases and treated with 4T1 cell and/or IL-12 therapy. Female CR:NIH-beige/nude/XID mice were inoculated with wild-type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse

such as Mig and IP-10 might be involved. To determine if our combination therapy stimulates Mig and/or IP-10 expression, RNA was prepared from the lungs of tumor-bearing, treated mice, reverse-transcribed, and PCR-amplified using Mig-specific and IP-10-specific PCR primers. To semi-quantify the amount of chemokine, PCR was performed for 26, 28, or 30 cycles. Although IP-10 was not expressed in the lungs (data not shown), Mig mRNA is detectable by PCR in the lungs of 4T1 tumor-bearing mice 4 h, 7 days and 21 days after initiation of therapy (Fig. 5C). Reverse transcription (RT)/PCR using β -actin primers confirmed the integrity of the RNA from treated mice (Fig. 5B). In contrast, lungs from untreated, tumor-free naive mice only showed a very faint band for Mig after 30 cycles, demonstrating that they have much lower levels of Mig in their lungs (Fig. 5A). Since naive lung does not express high levels of Mig, it is possible that the metastatic 4T1 cells are responsible for producing the Mig.

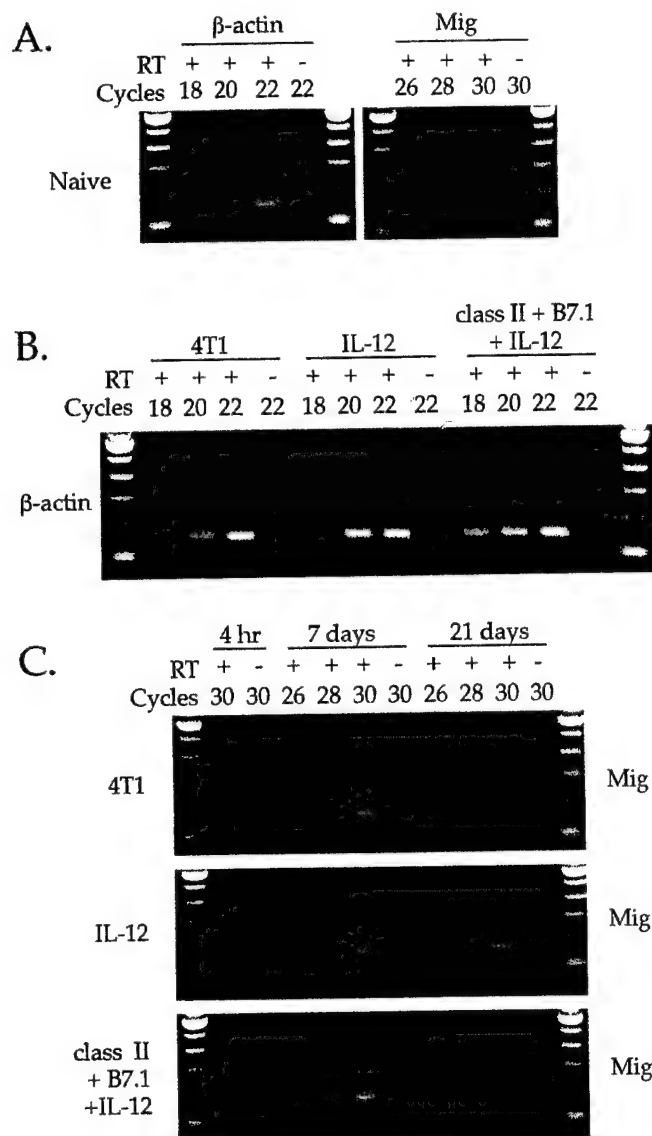


Fig. 5A–C mRNA for the monokine induced by interferon γ (Mig) is expressed in the lungs of BALB/c mice carrying 4T1 tumors. BALB/c mice were inoculated in the mammary gland with 7×10^3 4T1 cells on day 0 and therapy started on day 21. Lungs were removed 4 h, 7 days, and 21 days after therapy was started and lung RNA was isolated, reverse-transcribed, and amplified by the polymerase chain reaction using Mig-specific or β -actin-specific primers for the indicated number of cycles

To determine if the tumor cells synthesize Mig and therefore produce the Mig detected in the lungs, 4T1 and Mef10 cells were cultured with and without IFN γ for 2 h in vitro and Mig and β -actin expression analyzed by reverse PCR. As shown in Fig. 6, IFN γ induces expression of Mig in both 4T1 and Mef10 tumor cells. The resident tumor cells, therefore, may be generating the anti-angiogenic/chemoattractant factor.

Discussion

Several studies have reported a synergistic therapy effect when CD80-transfected tumor cells are combined with

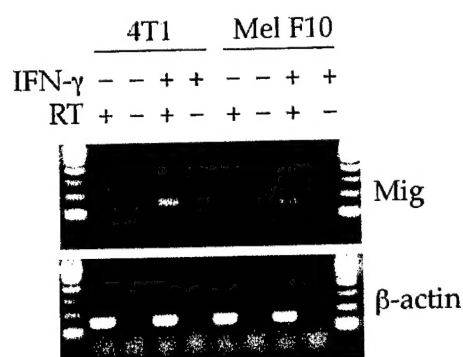


Fig. 6 4T1 and B16melF10 tumors are induced in vitro by interferon γ (IFN γ) to express Mig. RNA from 4T1 and melF10 tumor cells cocultured with or without IFN γ for 2 h was reverse-transcribed and amplified using Mig-specific or β -actin-specific primers

IL-12 and used as a vaccine to protect mice against a subsequent or simultaneous challenge of tumor [8, 9, 31, 45]. Other studies have shown therapeutic efficacy of CD80-transfected tumor cells combined with IL-12 in the treatment of very recently established experimental lung metastases [11, 34], or longer-established primary, solid tumors [33]. With the exception of one study, which reported that IL-12 up-regulated B7 expression on host antigen-presenting cells [14], all of these studies demonstrated more effective immunity when IL-12 and CD80 were combined.

Although all of these studies showed potent antitumor activity and reductions in tumor growth, only one of the reports examined spontaneous metastatic disease [22], and none of the reported studies addressed longer-term experimental or spontaneous metastatic disease. In addition, the previously reported studies have focused exclusively on activation of CD8 $^{+}$ T cells, although CD80 and IL-12 are potent activators of CD4 $^{+}$ T cells [23, 25]. Since much metastatic disease is poorly responsive to conventional treatments and immunotherapy is a potential alternative treatment, we have incorporated the combined therapy of CD80 plus IL-2 with our previously developed approach targeting the activation of CD4 $^{+}$ T cells, and tested the combined approach in two mouse

tumor systems in which metastatic lesions have been established longer and/or arise spontaneously.

In both the B16melF10 and 4T1 tumor systems the combination IL-12 plus cell-based vaccine therapy reduces metastases. The role of transfected tumor cells, however, may differ between the two tumors. For the B16melF10 tumor, therapy with IL-12 plus MHC class II, CD80-transfected tumor cells may provide greater protection than therapy with IL-12 plus non-transfected tumor cells. Although the increased protection is clearly apparent from visual inspection of the lungs of treated mice (Fig. 2), analysis of lung weights, using the appropriate statistical formula, does not demonstrate a significant difference between the groups treated with IL-12 alone and those receiving IL-12 plus cells. Lung weight is only an approximation of metastatic content, however, and it is unlikely that small, but significant, differences in numbers of metastatic cells will be detected by this measurement. Lung weights, therefore, probably do not accurately measure metastatic cells. Although enumerating the number of metastatic nodules in the lungs could also quantify metastatic spread, this measurement is also unlikely to reflect small, but significant differences, in the number of metastatic cells since individual nodules are of different size and contain different numbers of cells. The differences seen in Fig. 2, between the group receiving IL-12 alone and those treated with IL-12 plus vaccines, therefore, are likely to represent genuine differences in therapeutic efficacy of the different combinations of therapeutic agents even though the statistical analysis of lung weights does not indicate that these differences are significant. For the 4T1 tumor, treatment of established metastases with IL-12 plus any transfectant or wild-type tumor is equally efficacious, and the combination therapy of IL-12 plus cells is significantly more effective than therapy with IL-12 or cells alone.

Although numerous other studies have assessed effects of IL-12 on primary or i.v. induced experimental metastases, few animal models are suitable for analyzing the effects of IL-12 on individual animals with both primary tumor and metastatic disease. As shown in Table 4 for the 4T1 tumor, although therapy with IL-12

Table 4 Summary of primary and metastatic 4T1 tumor growth in BALB/c mice treated with IL-12 plus cell-based vaccine. In the case of the primary tumor, mice are classified as responders, partial responders, or non-responders. The diameter of the primary tumor decreased in responding mice, remained unchanged in partially responding mice, and increased in non-responding mice. In the case

of metastases (*Mets*), the mice are classified as either responders or non-responders. Responding mice have 10 000 or fewer clonogenic metastatic tumor cells in their lungs; non-responding mice have more than 10 000 metastatic cells. Results for the *Cells + IL-2* group are pooled from groups treated with IL-12 plus 4T1/A d , 4T1/B7.1, or 4T1 tumor cells

Therapy	Responding plus partially responding mice (%) having a primary tumor diameter at start of therapy (mm) of:							
	0-1.99		2-3.99		4-5.99		≥ 6	
	Primary tumor	Mets	Primary tumor	Mets	Primary tumor	Mets	Primary tumor	Mets
4T1 cells	0	100	0	50	0	0	0	0
IL-12	100	100	71	83	25	43	0	0
Cells + IL-12	100	100	82	100	19	52	0	66

plus cells does not affect growth of primary tumors of more than 4 mm, this therapy still reduces growth of metastatic cells in mice with primary tumors of this size. The combined immunotherapy of cells plus IL-12, therefore, is effective against metastases even when the host animal has a primary tumor that does not respond to the therapy.

Since IL-12 stimulates Th-1 lymphocyte activity [18, 23, 25], our expectation was that both CD4⁺ and CD8⁺ T cells would be required for the immunotherapy effect. Contrary to this expectation, neither T cell population alone was uniquely responsible for the therapy effect. Because IL-12 also stimulates NK cell activity, it was also anticipated that increased NK activity may contribute to the therapeutic effect. Although splenocytes with an NK phenotype, as measured by immunofluorescence, were increased in treated mice, there was no concomitant increase in NK functional activity, as measured by *in vitro* NK assay in the mF10 system. However, mice depleted *in vivo* for NK cells in the 4T1 system have slightly higher levels of metastatic tumor, suggesting that NK cells are involved in the therapeutic effect. Further confounding the identification of the effector cells in the combination therapy is the observation that mice deficient in NK and T cells (CR:NIH-beige/nude/XID mice) remain at least partially responsive to the therapy in the 4T1 system, suggesting that effector cells/mechanisms other than T and NK cells (e.g. neutrophils, eosinophils, macrophages, anti-angiogenesis factors, etc.) may also be involved in the therapeutic effect.

As recently suggested by several other studies, IL-12 stimulates IFN γ production, which, in turn, stimulates expression of chemokines such as Mig that may either directly or indirectly affect tumor growth [12, 20, 40]. Mig and/or IL-12 could be affecting tumor growth by at least three mechanisms. (1) Because Mig is a chemo-attractant for T cells and NK cells [15] it may facilitate migration of these effectors to the lungs where they mediate tumor cell destruction. (2) Because of their anti-angiogenic activity, Mig and IL-12 may directly or indirectly limit tumor-mediated angiogenesis [5, 12, 15, 20, 36, 43]. (3) Because IL-12 causes tumor necrosis, the lung metastases may become necrotic [37]. Collectively, the antibody depletion, chemokine induction, and experiments with immunodeficient mice in this paper suggest that the combined IL-12 and cellular vaccine therapy induces a combination of effector cells and effector molecules, including T cells, NK cells, neutrophils, and chemokines that synergistically diminish growth of lung metastases.

Mice treated in these studies had extensive tumor burdens and metastatic disease, much more advanced than mice commonly used in experimental immunotherapy protocols. Although the precise mechanism and contributions of each effector cell type and/or factor responsible for the anti-therapeutic effect are unclear, the combined use of tumor cells/transfectants plus IL-12 produces a more potent antitumor effect than either IL-12 or tumor cells/transfectants alone and the combined

use of these reagents in clinical immunotherapy protocols should be considered.

Acknowledgements We very much appreciate the help of Drs. T. Armstrong and B. Bradley with the statistical analyses, and thank Ms. Sandy Mason for the excellent care given to our animals. IL-12 was generously supplied by Dr. Stan Wolfe of Genetics Institute, Boston, Mass.

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Personnel List

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